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The epidemiology of Coxiella burnetii in goats in Indiana

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THE EPIDEMIOLOGY OF *COXIELLA BURNETII* IN GOATS IN INDIANA

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For my overly curious and overly educated family, especially my loving husband and patient children. I would like to promise you that I'm done after this, but I don't know that I can make that commitment.

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ABSTRACT

Bauer, Amy E. Ph.D., Purdue University, December 2015. The Epidemiology of *Coxiella burnetii* in Goats in Indiana. Major Professor: George E. Moore.

Coxiella burnetii is an obligate, intracellular bacterium and the etiologic agent of the zoonotic disease Q fever. Through the presence of an environmentally resilient small cell variant, *C. burnetii* can persist outside of a host in the face of extremes in temperature, humidity, and pressure. *C. burnetii* is considered to be ubiquitous in the environment and endemic in cattle, sheep and goat populations. These same species are the main reservoirs for human infection with *C. burnetii*. The overall goal of this project was to develop a baseline understanding of the epidemiology of *C. burnetii* in goats in Indiana. Specific areas addressed were: Individual and herd level prevalence of infection with *C. burnetii* as evaluated by serologic and molecular methods, geographic distribution of individuals and herds positive for infection with *C. burnetii*, and evaluation of potential risk factors for infection with *C. burnetii* among individual goats and herds. Six-hundred-fifty-four does representing 95 herds were included in the study sample as a whole. Six-hundred-forty-nine of the does from 94 farms were from Indiana. Based on the use of a commercial enzyme-linked immunosorbent assay (ELISA), there was a 3.8% estimated individual level and 11.2% estimated herd level seroprevalence

for *C. burnetii* in Indiana. Through use of a real-time polymerase chain reaction (PCR) assay targeting the IS1111 transposon of *C. burnetii* in DNA samples from either milk, vaginal mucus samples or feces, there was a 7.5% estimated prevalence of shedding *C. burnetii* at the individual level and a 20.2% estimated prevalence of shedding *C. burnetii* DNA at the herd level. There was no statistically significant difference detected between regions of Indiana in regards to does testing positive for *C. burnetii*. However, a statistically significant association was detected between does testing positive for *C. burnetii* and the Public Health Preparedness District (PHPD) of Indiana in which the farm was located. Finally, 3 potential risk factors of interest: The presence of cattle, sheep or camelids on the farm; history of abortion, stillbirth or weak offspring within the herd; and whether or not the goats were housed primarily indoors were evaluated for associations with seropositivity for or shedding of *C. burnetii*. Of these 3 factors, only the presence of cattle, sheep or camelids on the farm demonstrated a statistically significant association with the likelihood of detecting either anti-*C. burnetii* antibodies or shedding of *C. burnetii* DNA. Rather than increasing the likelihood of detecting *C. burnetii*, the presence of these other species on the farm appears to have a protective effect.

CHAPTER 1. INTRODUCTION

Coxiella burnetii is the causative agent of Q fever, a disease that has varying manifestations among and within species, some of which can be both life threatening and emotionally devastating. With a low infectious dose and transmission through inhalation of aerosols, *C. burnetii* has been researched as a potential bioweapon and is currently a Class B bioterror agent (Oyston & Davies, 2011). Working with the live agent requires Biosafety Level 3 precautions. Although precautions should be taken when working with *C. burnetii* in a laboratory setting, it is presumed to be endemic in cattle, sheep and goats worldwide and ubiquitous in the environment. Indeed the presence of *C. burnetii* has been documented in environmental dust samples from post offices, high schools and grocery stores based upon the detection of the insertion sequence 1111 transposon (IS1111) by quantitative polymerase chain reaction (PCR) (Kersh et al, 2010).

Q fever has been a reportable disease in the United States since 1999 (McQuiston et al., 2006) and is reportable in both people and animals in Indiana. The Centers for Disease Control and Prevention (CDC) reported an increasing trend in the diagnosis of Q fever in the United States between 2000 and 2007 with a range from 17 to 167 cases

reported annually (CDC, 2013). A study conducted between 2003 and 2004 tested 4,437 people for anti-*C. burnetii* antibodies and estimated a national seroprevalence of 3.1% (Anderson et al, 2009). The incidence rate for Q fever between 2000 and 2012 was 0.38 cases per million people per annum (Dahlgren et al, 2015). In Indiana, a peak number of human Q fever cases occurred in 2005 with 4 cases reported to the Indiana State Department of Health (Beall et al., 2007).

The presence of *C. burnetii* in cattle from Indiana has been documented in studies at the national level (Kim et al, 2005; APHIS, 2011). Of 316 bulk tank milk samples from commercial cattle dairies collected by the Indiana State Board of Animal Health in 2011, 193 (60.8%) tested positive for the presence of the IS1111 transposon of *C. burnetii* (Bauer et al, 2015). Cattle are only 1 of the host species capable of transmitting *C. burnetii* to people. Sheep and goats are also reservoir species for *C. burnetii* (Norlander, 2000). In recent years, goats have been more likely to be reported as the source of human outbreaks of Q fever (Bamberg *et al.*, 2007; Schimmer *et al.* 2010; Bjork *et al.*, 2014).

The overarching goal of the studies reported herein was develop a baseline understanding of the epidemiology of *C. burnetii* in goats in Indiana. The studies reported in the following chapters do not seek to completely define the epidemiology of *C. burnetii* in Indiana, but rather to lay a foundation for future work that enhances the understanding of the role of goats in the epidemiology, ecology, and public health of *C. burnetii* and Q fever.

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CHAPTER 2. LITERATURE REVIEW

2.1 Taxonomy of *Coxiella burnetii*

Coxiella burnetii is a small, gram negative, intracellular coccobacillus (Oyston & Davies, 2011). It is taxonomically classified in the phylum *Proteobacteria*, class *Gammaproteobacteria* and order *Legionellales* which it shares with the family *Legionellaceae*. In addition to *Coxiella*, the *Coxiellaceae* family includes the genera *Rickettsiella*, *Diplorickettsia*, and *Aquicella* (Drancourt & Raoult, 2005). A second proposed species, *Coxiella cheraxi*, has been identified in freshwater crayfish (Tan & Owens, 2000). In addition to *C. burnetii* and *C. cheraxi*, *Coxiella*-like endosymbionts have been documented in several species of ticks (Klyachko et al., 2007; Liu et al., 2013; Wilkinson et al., 2014), though species status has not yet been proposed for these organisms.

Prior to 2009 and the development of specialized media mimicking the intracellular environment in which it reproduces, *C. burnetii* was not able to be cultured in vitro (Omsland et al., 2009). Because of this, identification of different strains of *C. burnetii* has been based on molecular techniques. Several methods have been utilized to define genotypes of *C. burnetii*. Historically these included restriction length fragment

polymorphism analysis (RFLP) (Jager et al., 1998), plasmid typing and comparison of the molecular sequences of highly conserved genes such as *com-1*, which codes for an outer membrane protein (Hendrix et al., 1993), and isocitrate dehydrogenase (*icd*) (Massung et al., 2012). More recently, high resolution techniques have been developed including: Multilocus variable tandem number repeat analysis (MLVA) (Roest et al., 2011), multispacer sequence typing (MST) (Glazunova et al., 2005), and whole genome comparisons based upon microarray technology (Massung et al., 2012).

RFLP analysis is based upon differences in patterns in bands when viewing gel electrophoresis or Southern blot products. Restriction enzymes targeting specific nucleotides at each end of the sequence of interest are used to cut DNA into fragments. Changes in the target sequence will change the size and number of the fragments, resulting in different patterns of banding on agarose gel electrophoresis (Tang et al, 1997; Jager et al., 1998). Utilizing PCR-RFLP, Nguyen and Hirai were able to divide 19 isolates of *C. burnetii* into 3 groups. Isolates from acute Q fever patients clustered with samples from cattle and isolates from chronic Q fever patients clustered with a sample from a goat with an aborted pregnancy (Nguyen & Hirai, 1999).

Plasmid typing of *C. burnetii* focuses on the 5 types of plasmids identified in various *C. burnetii* genomes. Four of these are true plasmids (QpDG, QpDV, QpH1 and QpRS) and one is a plasmid-homologous sequence incorporated into the chromosome (Massung et al., 2012). These 5 plasmids defined 5 different genomic groups of *C. burnetii*, some having specific geographic associations (Massung et al., 2012).

Highly conserved genes may display random sequence variations that can be utilized for genotyping. The *com-1* and *icd* genes are most commonly chosen for this type of analysis. *Com-1* codes for an outer membrane protein (Hendrix et al., 1993). Four to 5 different genogroups have been identified based on analyses of variation in the *com-1* sequence (Zhang et al, 1997; Massung et al., 2012). *Icd* codes for a dimeric NADP(+)-dependent isocitrate dehydrogenase similar to that of *E. coli* and *S. enterica*, but is preferentially expressed at a low pH such as that favored by *C. burnetii* (Nguyen et al., 1999). Based on *icd* analysis, 3 different genogroups of *C. burnetii* have been identified (Massung et al., 2012). Poor discriminatory power has resulted in the abandonment of these methods of genotyping (Massung et al., 2012) although these genes are still used in diagnostic PCR.

MLVA is based upon calculating the number of small repeats within a target region or regions of a bacterial genome. MLVA has a high discriminatory power, but there are challenges in defining the most common sequence pattern (consensus sequence) for reference between laboratories (Massung et al., 2012). Nevertheless, MLVA has been frequently used to identify genotypes of *C. burnetii* (Arricau-Bouvery et al., 2006, Boarbi et al., 2014, Frangoulidis et al., 2014).

MST identifies genetic sequences, 300 to 700 base pairs in length, positioned between coding regions of the genome. In comparison to MLVA, MST analysis has a lower discriminatory power but MST results are more easily compared between groups (Massung et al., 2012). Using MST, 30 different sequence types (ST) of *C. burnetii* were initially described and STs 8, 16, 20 and 21 were identified in samples from the United

States (Glazunova et al., 2005). In addition to geographic trends, STs 1, 4, 16 and 18 were associated with human patients that presented with acute Q fever and ST8 was associated with human patients presenting with chronic Q fever (Glazunova *et al.*, 2005). As application of MST has continued, additional sequence types have been identified, including a novel form from French Guiana, ST17, which may have the 3-toed sloth as its primary reservoir (Mahamat et al., 2013, Davoust et al., 2014). This strain is associated with a particularly severe form of acute Q fever with a higher prevalence of pneumonia in infected patients when compared with acute Q fever patients in France (Edouard *et al.*, 2014). A single nucleotide polymorphism (SNP) analysis has been developed to differentiate between MST sequence types (STs) of *C. burnetii* (Hornstra et al., 2011). SNP analysis targets a change in nucleotide at a single site in the genetic region of interest. Utilizing this analysis, ST8 was identified as the most common ST in goat milk in the United States, although ST20 was also present in some samples (Pearson et al., 2014).

2.2 Lifecycle of *Coxiella burnetii*

C. burnetii has a complex lifecycle involving an intracellular, metabolically active form called the large cell variant (LCV) and two apparently metabolically inactive forms that are resilient enough to survive in the environment called the small cell variant (SCV) and small dense cell (SDC) (Norlander, 2000). Large cell variants may also contain polar endospore-like structures, the role of which is unknown (Heinzen et al., 1999; Minnick & Raghavan, 2012). These are not considered to be true endospores because the *C. burnetii* genome does not contain the genes normally associated with sporulation and

does not produce dipicolinic acid (Minnick & Raghavan, 2012). In *Bacillus subtilis* spores, the presence of dipicolinic acid is associated with resistance to damage from wet heat and protection of DNA from damage due to light, dry heat and desiccation (Setlow *et al.*, 2006). The SDC is a laboratory observed variant of the SCV that is produced when *C. burnetii* is treated with pressure in excess of 20,000 pounds per square inch (Minnick & Raghavan, 2012). The resilient, metabolically inactive SCV is the infectious form of *C. burnetii* and allows the organism to persist in a contaminated environment, leading to increased exposure of susceptible hosts to the bacterium.

Differences in the physical structure and protein expression between the SCV and LCV can be related to different requirements for environmental resilience as compared to active metabolism and reproduction. The SCV is, as its name implies, the smaller of the two forms, measuring approximately 0.2-0.5μm. The genetic material in the SCV is condensed and an intracellular membrane system is also present. LCVs are greater than 1μm in length and pleomorphic with dispersed genetic material. Aside from physical differences, certain proteins are differentially expressed in the SCV and the LCV (Minnik and Raghaven, 2012). Minnik and Raghavan refer to these as SCV^{Hi}/LCV^{Lo} and LCV^{Hi}/SCV^{Lo} respectively. SCV^{Hi}/LCV^{Lo} proteins are important promotors of cell differentiation, substrate uptake, and condensation of genetic material (Minnick & Raghavan, 2012). LCV^{Hi}/SCV^{Lo} proteins include those that promote translation, help to mediate the environmental challenges of replicating in a highly acidic environment,

manage transcription and aid both DNA replication and cell division. Both groups contain proteins that maintain biosynthetic pathways (Minnick & Raghavan, 2012).

In addition to the SCV and LCV, two phases of *C. burnetii* exist based upon the lipopolysaccharide content of the cell wall. Phase I bacteria have a complete lipopolysaccharide surface while phase II bacteria have a truncated lipopolysaccharide lacking the O antigen (Voth & Heinzen, 2007; Oyston & Davies, 2011). The genes coding for this antigen may in fact be absent in some phase II *C. burnetii*. Phase II bacteria are generally not identified in vivo, but develop after serial passages in tissue culture or embryonated eggs (Voth & Heinzen, 2007; Oyston & Davies, 2011). It was initially hypothesized that the phase of the bacteria related to the form of clinical disease such that infection with phase I bacteria resulted in acute Q fever while infection with phase II resulted in the chronic form, but this model is not currently accepted (Heinzen et al., 1999). Rather, phase I bacteria are the virulent form of *C. burnetii* while phase II bacteria are avirulent in immunocompetent individuals (Voth & Heinzen, 2007; Shannon & Heinzen, 2009).

Once inhaled or ingested by the host, the phase I SCV preferentially targets macrophages, particularly in the lung and liver. The complete phase I LPS likely acts to hide surface antigens from pattern recognition receptors on dendritic cells (Shannon & Heinzen, 2009). Lack of dendritic cell response to *C. burnetii* affects the response of macrophages to the organism. Rather than expressing M1 polarization, a microbicidal response, macrophages instead respond to phase I *C. burnetii* with M2 polarization. This results in a lower rate of phagocytosis and lack of microbicidal activity (Ben Amara et al.,

2012). Phase I *C. burnetii* uptake by TLR4 in M2 macrophages involves only the $\alpha v\beta 3$ integrin which leads to cytoskeletal reorganization. This reorganization results in cell spreading and the formation of filopodia and lamellopodia by the macrophages due to underlying localization of F-actin (Honstettre et al., 2004). Uptake of phase II *C. burnetii*, where dendritic cells are activated and macrophages produce an M1 response, is mediated by both $\alpha v\beta 3$ and CR3 integrins and produces a greater rate of phagocytosis (Ben Amara et al., 2012). Indeed, when phagocytosis indices (PI) of *C. burnetii* with phase I LPS were compared between wild type and TLR4 deficient mice, the TLR4 deficient mice had a lower mean PI than the wild type mice (Honstettre et al., 2004). PIs were also compared between phase I and phase II *C. burnetii*. Phagocytosis indices were greater for *C. burnetii* with phase II LPS as compared to *C. burnetii* with phase I LPS, but there was not a statistically significant difference in these measures between wild type and TLR4 deficient mice for phase II LPS (Honstettre et al., 2004). The role LPS plays in inhibition of the uptake of *C. burnetii* was also evaluated in this study. When polymyxin B was used to block binding of LPS by monocytes in vitro, organisms exhibiting phase I LPS were blocked from uptake, while those exhibiting phase II LPS were not blocked (Honstettre et al., 2004). Thus, the complete phase I LPS appears to aid *C. burnetii* in inhibiting the immune response without completely suppressing the phagocytic process necessary for its incorporation into macrophages for replication.

After phagocytosis, *C. burnetii* stalls the maturation of the phagosome for approximately 2 hours. A second virulence factor, the type IV Dot/ICM secretion system

(T4SS) is translocated about 8 hours after phagocytosis (van Schaik et al., 2013). Type IV secretion systems help to control the development of infection over time and the *C. burnetii* T4SS potentially utilizes 22 different effectors to promote infection and replication (van Schaik et al., 2013). These effectors localize to sites such as the Golgi apparatus, the nucleus, the autophagosome and the mitochondrion and may be involved in how *C. burnetii* controls the secretory pathway, transcription and apoptosis in the host cell (van Schaik et al., 2013). Over a 1 to 2 day lag period, the SCV of *C. burnetii* transform to LCV and the phagosome becomes an early parasitophorous vacuole (PV) with a cholesterol-rich plasma membrane and an internal pH of 4.5-5 (Howe & Heinzen, 2005; Minnick & Raghavan, 2012; Gilk, 2012). The large cell variants then divide by binary fission during a log phase of growth (Ghigo et al., 2012). Approximately 4-6 days postinfection, the PV contains extensive numbers of LCVs, and enters the stationary phase of replication. The LCVs begin to transition back to SCVs during this phase (Voth & Heinzen, 2007). In tissue culture, rupture of the PV and the cell in which it has become the dominant structure at approximately 12-16 days post-infection releases the SCVs (Minnick & Raghavan, 2012). Because *C. burnetii* can exert control over apoptosis, not all of the infected cells rupture. In fact, infected cells can divide such that the PV is localized into a single daughter cell (Minnick & Raghavan, 2012).

Replication of *C. burnetii* cannot occur without the formation of a PV. Inhibition of cholesterol metabolism by agents such as ketoconazole, imipramine and progesterone has been demonstrated to disrupt PV formation through altered

cholesterol metabolism (Howe & Heinzen, 2005). These agents do not act uniquely on the cholesterol biosynthetic pathway, but rather affect general sterol production, clouding the importance of cholesterol specifically for *C. burnetii* replication as compared to sterols in general (Gilk, 2012). Future treatments for Q fever may focus on the inhibition of cholesterol metabolism and other methods to block PV formation.

Although *C. burnetii* makes use of the phagolysosome within macrophages for replication, the immune system is not completely deceived by the pathogen and both cell-mediated and humoral immune responses to infection occur. Lymphocyte production of interferon gamma leads to recruitment of fibroblasts, additional macrophages, and monocytes as well as the production of reactive oxygen and nitrogen species (Shannon & Heinzen, 2009). T-cells play a role in controlling acute *C. burnetii* infection, but at this time the specific type of T-cell involved in the cell-mediated response is undetermined (Shannon & Heinzen, 2009). There is some evidence that regulatory T-cells may play a role in the persistence of *C. burnetii* within the body and thus the development of the chronic form of Q fever (Ben Amara et al., 2012). The humoral response to acute infection by *C. burnetii*, characterized by high levels of antibody responding to the phase II LPS, occurs within 3 to 4 weeks of the development of clinical signs (Shannon & Heinzen, 2009). Antibody opsonization of phase I *C. burnetii* appears to increase phagocytosis by macrophages, but does not affect intracellular growth; in contrast, opsonized phase II *C. burnetii* stimulate maturation and production of inflammatory cytokines by dendritic cells (Shannon & Heinzen, 2009).

The immune response in chronic *C. burnetii* infection has been associated with increased levels of interleukin-10 (IL-10) (Shannon & Heinzen, 2009). These increased levels may be related to immunosuppression in the chronic form of Q fever (Capo & Mege, 2012). Interestingly, IL-10 may also play a role in the observed higher level of Q fever diagnosis in men as compared to women. In evaluating gene modulation in *C. burnetii* infected mice, 60% of the observed modulations were related to sex hormones with the modulation expressed to greater degree in males than in females. IL-10 genes are among those upregulated in males (Capo & Mege, 2012).

2.3 Transmission of *Coxiella burnetii*

C. burnetii transmission has been documented to occur by four routes: Inhalation, ingestion, sexual contact and tick bite. The most important of these routes for human and domestic animal disease is inhalation. Ingestion of *C. burnetii*, particularly through consumption of unpasteurized milk, is of public health concern, but the magnitude of this concern is debatable. Sexual transmission has been reported in the spouses of military servicemen returning from the Middle East to the United States (Miceli et al., 2012) and the potential for transmission in embryo transfer materials has also been studied (Alsaleh et al., 2011). Transmission by tick bite is believed to play an important role in maintaining circulation of *C. burnetii* in wildlife populations, but further research is needed to clarify the role of arthropod vectors in domestic animals and people.

Inhalation is the most important route for transmission of *C. burnetii*. Organismal DNA has been identified in particles small enough for inhalation (Hogerwerf et al., 2012).

Inhalable dust samples (particulate size 50% cutoff of 30μm) were captured in glass fiber filters with a diameter of 37mm and smaller, PM10 particles (diameters of 10μm or less) were captured using Teflon filters with a 2.0μm pore size at farms where goats had been culled due to a Q fever outbreak. *C. burnetii* was identified in both the PM 10 and the larger, inhalable particles. The percentage of airborne particles positive for *C. burnetii* was greater in samples collected during periods of activity on the farms. Settled dust samples were also collected and 13 of the 14 collected samples were positive for the presence of *C. burnetii* (Hogerwerf et al., 2012). *C. burnetii* was also found to persist in air samples taken 1 year after an outbreak of Q fever abortion in goats (Kersh et al., 2013), indicating that while resolution of clinical disease may reduce the risk of transmission of *C. burnetii*, the risk is not eliminated.

Transmission of *C. burnetii* through ingestion has been a concern almost since the discovery of the organism. Although pasteurization was initially developed to help control brucellosis, the recommended temperature settings were developed to inactivate *C. burnetii* (Cerf & Condron, 2006). Milk is the main route of shedding of *C. burnetii* in dairy cattle (Rodolakis et al., 2007), and prevalence estimates of herds shedding *C. burnetii* in milk are high (Kim et al., 2005; APHIS, 2011). Outbreaks of *C. burnetii* have been linked to consumption of unpasteurized dairy products (Fishbein & Raoult, 1992; Signs et al., 2012). However there is debate as to the epidemiologic importance of the oral route of transmission and indeed whether *C. burnetii* should be considered to be a food-borne pathogen (Cerf & Condron, 2006; Gale et al., 2015).

Sexual and reproductive contacts are also potential routes of transmission of *C. burnetii*. In humans, cases of Q fever have been linked to sexual activity (Miceli et al., 2012) as well as childbirth (Raoult & Stein, 1994). *C. burnetii* has been identified in bull semen (Kruszewska & Tylewska-Wierzbanowska, 1997). Embryo transfer has also been identified as a potential route of *C. burnetii* transmission in domestic ruminants. *C. burnetii* DNA was identified in 8/20 sample of flushing media collected from caprine reproductive tracts in a simulation of the embryo harvesting process (Alsaleh et al., 2011). More work is needed to evaluate artificial insemination and embryo transfer as routes of *C. burnetii* transmission between conspecifics, but there is certainly the potential for organism transfer by these routes.

Finally, tick bites are considered to be a route of *C. burnetii* transmission, though this is likely more important in wildlife than in people. Experimental work has documented transmission of *C. burnetii* to guinea pigs by tick bite (Smith, 1941), but there have been few case reports of Q fever linked to ticks. A case reported in 1947 reported interactions with ticks, “Ticks were numerous and those removed from his clothes were destroyed by being crushed with his fingers,” but no bites (Eklund et al., 1947). A study of 400 confirmed cases of Q fever identified concurrent infection with other known tick-borne pathogens in 6 individuals, 3 of which were believed to be due to simultaneous infection after a tick bite (Rolain et al., 2005). While ticks should not be ruled out as a reservoir for *C. burnetii* in wildlife and domestic animals, their role in the spread of *C. burnetii* to people is likely secondary to that of other routes of transmission.

Close association with infected individuals or products from infected animals is not necessary for exposure to *C. burnetii*. Wind can carry the bacteria away from the original infected location (Tissot-Dupont et al., 1999,; Tissot-Dupont et al., 2004; Arricau-Bouvery & Rodolakis, 2005; van der Hoek et al., 2010; Smit et al., 2012). Indeed, windborne transmission of *C. burnetii* from farms experiencing abortion storms has been identified as a factor in the largest modern outbreak of human Q fever (Schimmer et al., 2010) as well as smaller outbreaks (Tissot-Dupont et al., 1999). In a population-based study in the Netherlands, the presence of greater than 2,250 goats within 5km of a residence was associated with an increased odds of diagnosis of "other infectious disease", a morbidity classification which is defined as Lyme disease or Q fever in the Netherlands (Smit et al., 2012). The odds of diagnosis with "other infectious disease" increased with animal density, with people living within 5km of 2,251-7,250 goats demonstrating a 2-fold increase in the odds of this diagnosis, those living within 5km of 7,251-17,190 goats demonstrating a 4 fold increase in the odds of this diagnosis and those living within 5km of 17,191-20,960 goats demonstrating a 12-fold increase in the odds of this diagnosis when compared with individuals living within 5km of 2,250 or fewer goats (Smit et al., 2012). Dry conditions also appear to be linked with an increased incidence of clinical disease (Tissot-Dupont et al., 1999; Tissot-Dupont et al., 2004; van der Hoek et al., 2010). The presence of *C. burnetii* has been documented in locations where the presence of animals would not be expected such as high schools, banks and post offices. Environmental dust samples were collected by HEPA vacuum, sponge or swab wipes from solid surfaces or by collecting 10-50g of ground material (soil). At some

sites, up to 50% of the samples tested demonstrated the presence of *C. burnetii* DNA (Kersh et al., 2010). The ubiquity of *C. burnetii* becomes important when considered in its context as a potential bioweapon (Oyston & Davies, 2011). Knowledge of the prevalence and strains of *C. burnetii* naturally circulating within ruminant populations and present in the environment is an important consideration in differentiating natural outbreaks from biological attacks.

2.4 *Coxiella burnetii* host species

Small ruminants are the main reservoir species for human infection with *C. burnetii* (Hugh-Jones et al., 1995; Rodolakis et al., 2007). This may be due to the importance of bacterial shedding in feces and urine in these species, which result in greater contamination of the environment (Arricau-Bouvery & Rodolakis, 2005). Alternately, the strain(s) of *C. burnetii* infecting small ruminants may be more virulent, leading to a larger number of Q fever diagnoses related to the these species as compared to cattle.

C. burnetii is considered to be enzootic in all domestic ruminants in the United States and ubiquitous worldwide with the apparent exception of New Zealand (McQuiston et al., 2006; Hilbink et al., 1993; Greenslade et al., 2003). There may be no manifestation of disease in cattle, sheep or goats. However, abortion, stillbirth and weak offspring can result from infection in all of these species. When abortion or stillbirth occurs, *C. burnetii* is shed in great numbers in the placenta and reproductive discharges, contaminating the environment (Norlander, 2000; Oyston & Davies, 2011). Fecal and urine shedding have been documented in sheep and goats and are important routes of

shedding and sources of environmental contamination in some species (Arricau-Bouvery & Rodolakis, 2005; Oyston & Davies, 2011). *C. burnetii* is also shed in milk, particularly by cattle and goats, and the possibility of transmission through ingestion of unpasteurized milk products is present (Fishbein & Raoult, 1992; Rodolakis et al., 2007; Arricau-Bouvery et al., 2003).

In addition to cattle, sheep and goats, multiple species, across several taxa, are able to become infected with and transmit *C. burnetii*. Among domestic species, both the domestic cat (Pinsky et al., 1991) and the domestic dog (Buhariwalla et al., 1996) have been associated with outbreaks of Q fever. *C. burnetii* has also been identified in chickens, quail and domestic waterfowl (To et al., 1998) as well as the domestic camel (Rahimi et al., 2011; Mohammed et al., 2014).

Infection with *C. burnetii* has been investigated in wildlife species closely associated with human beings. Pigeons were implicated in a French outbreak of Q fever (Stein & Raoult, 1999). Seropositivity for *C. burnetii* has been documented in wild rats in India (Yadav et al., 1979), the United Kingdom (Webster et al., 1995) and the Netherlands (Reusken et al., 2011). A 2011 review identified worldwide reports of seropositivity for exposure to *C. burnetii* in 7 species of rat and 9 species of mouse. Isolation of *C. burnetii* was reported in 4 species of rat and 4 species of mouse. The isolation studies were all published prior to 1980, thus modern molecular techniques were not employed (Meerburg & Reusken, 2011).

Numerous wildlife species that are not generally associated with people have also been documented as hosts of *C. burnetii*. Prevalence estimates have been made for

multiple species of deer and moose in various geographic regions (Marrie et al., 1993; Rijks et al., 2011; Ohlson et al., 2014; Shin et al., 2014,). For deer species, seroprevalence ranged from 1.5% in white tailed deer in Nova Scotia (Marrie et al., 1993) to 9.2% in Korean water deer (Shin et al., 2014). Detection of *C. burnetii* DNA in deer ranged from 6.6% in Korean water deer (Shin et al., 2014) to 23% in roe deer in the Netherlands (Rijks et al., 2011). It is of note that the latter study was conducted during an outbreak of Q fever in dairy goats and people (Rijks et al., 2011). In moose, no seropositive individuals were detected among 99 sampled in Sweden (Ohlson et al., 2014) while 16.5% of the individuals sampled in Nova Scotia had detectable antibodies (Marrie et al., 1993). Marsupials are also known to be hosts for *C. burnetii* (Bennett et al., 2011; Cooper et al., 2012) although there have not been reports linking these species to human infection. Conversely, the three-toed sloth has been proposed as the reservoir species for a strain of *C. burnetii* that caused severe Q fever in French Guiana (Davoust et al., 2014). Infection with *C. burnetii* has also been reported in marine mammals (Kersh et al., 2010) and the environment of their calving grounds (Duncan et al., 2012). This raises the concern of transmission of *C. burnetii* to individuals harvesting and consuming the meat of marine mammals, but at this time there have been no reports of transmission by this route (Tryland et al., 2014).

Ticks have been well documented to carry both *C. burnetii* and *Coxiella*-like symbiotes. Species of ticks documented as carriers of *Coxiella* include *Amblyomma americanum* and *Dermacentor silvarum* (Klyachko et al.; 2007, Liu et al., 2013). Ticks are believed to maintain infection with *C. burnetii* in wildlife species, but given the

challenges of working with *C. burnetii*, this hypothesis has mostly been explored through observational studies involving collecting ticks and using molecular techniques to identify the symbiotic microbes (Klyachko et al., 2007; Liu et al., 2013).

2.5 Q fever: Clinical disease and outbreaks

Q fever is the clinical form of infection with *C. burnetii*, although the term coxiellosis has also been used for clinical infection in non-human species. It is assumed that the majority of *C. burnetii* infections in animals are asymptomatic. Among the domestic ruminant reservoirs of *C. burnetii*, the clinical presentation of Q fever can vary by species. Abortion and stillbirth due to *C. burnetii* have been reported to occur in cattle, but there has been no experimental confirmation of causality (Garcia-Ispierto et al., 2014). Similarly, retained placenta, mastitis, metritis and endometritis have all been linked to infection with *C. burnetii* in cattle, but evidence to support these associations is lacking (Garcia-Ispierto et al., 2014). In comparison to cattle, small ruminants, particularly goats, are more likely to exhibit reproductive effects from *C. burnetii* infection, including late term abortion, stillbirth or weak kids (Arricau-Bouvery & Rodolakis, 2005). In experimental infection of 12 goats by subcutaneous injection at 90 days of pregnancy, mild hepatitis and interstitial pneumonia were identified in 2 does sacrificed at days 26 and 40 after infection but prior to abortion (Sanchez et al., 2006). Inflammatory changes were also identified in the mammary gland of 1 of 2 does sacrificed on day 40. The remaining infected does all aborted, but no pathology was identified in organs other than the maternal placenta at the time of sacrifice and necropsy (Sanchez et al., 2006).

Although serology is an important method of screening for the presence of *C. burnetii* within a herd, seroconversion in goats has been evaluated in few studies. An experimental study and a case-control study in the context of an outbreak of Q fever have evaluated the relationship between abortion and the detection of anti-*C. burnetii* antibodies in goats (Arricau-Bouvery et al., 2003; Rousset et al., 2009). In the experimental study pregnant does were infected by subcutaneous injection with a strain of *C. burnetii* known to be associated with abortion in goats (CbC1). The administered doses ranged from 10^4 to 10^8 organisms (Arricau-Bouvery et al., 2003). None of the does tested positive by enzyme-linked immunosorbent assay (ELISA) until day 42 after infection and only 67% were seropositive at the time of abortion. By 80 days after infection, 91% of the does had seroconverted. Does were sacrificed at 6 weeks post-abortion so the duration of the immune response was not determined (Arricau-Bouvery et al., 2003). Fifty does that experienced abortion and 70 does with normal parturition were recruited from 8 goat dairies where at least 5 does in the herd had aborted due to Q fever (Rousset et al., 2009). The recruitment goal for each dairy was 15 does: Five that had aborted, 5 at full term gestation and due to freshen and 5 that were in the last month of gestation. However, 10 of the control does aborted and were included as cases. These does had all been infected by a natural route and the strain of *C. burnetii* was not reported. Paired serum samples were collected from the does at 15 and 30 days after either the peak of abortion at the farm, the last month of gestation or parturition. Eighty-nine of the does tested positive for exposure to *C. burnetii* by ELISA (Rousset et al., 2009). The number of does that seroconverted between day 15 and day 30 was not

reported and follow-up beyond 30 days was not performed, so duration of the immune response could not be determined.

In people, the acute form of Q fever was first described in slaughterhouse workers whose positions included interaction with cattle, sheep or their products including hides (Derrick, 1937). Q fever is still considered to be an occupational disease among those who work with livestock (Behymer & Reimann, 1989; Whitney et al., 2009). Based on the comparison of the number of seropositive individuals with symptomatic individuals, asymptomatic Q fever is believed to be common in the general population (Norlander, 2000). The Centers for Disease Control and Prevention estimates that 3% of the general population of the United States is serologically positive for *C. burnetii* exposure (CDC, 2013). The symptoms of acute Q fever are influenza-like, including a fever ranging from 102.0 to 104.0^{o}F associated with headache. These symptoms last from 7 to 24 days. The clinical similarity to influenza complicates diagnosis and without a high index of suspicion Q fever can easily be misdiagnosed (Derrick, 1937; Norlander, 2000; Arricau-Bouvery & Rodolakis, 2005). Complications of acute Q fever can include hepatitis, either asymptomatic or granulomatous with hepatomegaly, pneumonia and encephalitis (Norlander, 2000; Arricau-Bouvery & Rodolakis, 2005).

Chronic Q fever may develop in people as a sequelae to acute infection. Endocarditis and a form of chronic fatigue syndrome are the most common manifestations of chronic Q fever (Harris et al., 2000; Norlander, 2000; Arricau-Bouvery & Rodolakis, 2005; Oyston & Davies, 2011). Q fever endocarditis is most likely to develop in those with pre-existing cardiac abnormalities or immunosuppressive

conditions (Arricau-Bouvery & Rodolakis, 2005). Post-Q fever fatigue syndrome occurs in approximately 15% of people diagnosed with acute infection and can linger for up to a decade after the initial infection with *C. burnetii* (Harris et al., 2000, Oyston & Davies, 2011). Chronic Q fever results in higher mortality rates than the acute form (Norlander, 2000).

Abortion related to placentitis and other reproductive effects of *C. burnetii* infection also occur in women. The active *C. burnetii* infection does not have to occur concurrently with pregnancy for reproductive effects such as premature birth, low birth weight, neonatal mortality and repeated miscarriages to occur (Norlander, 2000, Arricau-Bouvery & Rodolakis, 2005). In a series of 53 cases of pregnant women diagnosed with Q fever during pregnancy between 1991 and 2005, only 18.9% of women infected with *C. burnetii* had a normal pregnancy outcome. Pregnancy complications, in descending order of frequency, were: Premature delivery, stillbirth, intrauterine growth restriction, spontaneous abortion and oligoamnios (Carcopino et al., 2009). In addition, pregnant women have a higher risk of developing chronic Q fever, particularly when infection occurs early in pregnancy (Carcopino et al., 2009).

Whether in animals or people, much of the research on the epidemiology of *C. burnetii* has been performed in the context of outbreaks of Q fever (Fishbein & Raoult, 1992; Tissot-Dupont et al, 2004; Rousset et al, 2009; Schimmer et al, 2010; Reusken et al, 2011; Rijks et al, 2011; Roest et al, 2011; Hogerwerf et al, 2012; Kersh et al 2013; Mahamat et al, 2013). Within the past 10 years, 2 outbreaks of note have involved goats

as the source of human infection. Although different in scale, both of these outbreaks have contributed to the understanding of the epidemiology of *C. burnetii*.

In May of 2007, an outbreak of Q fever associated with disease in dairy goat and sheep farms was documented in the area surrounding a village in the southeastern region of the Netherlands (van der Hoek et al., 2010). This developed into one of the largest documented Q fever outbreaks in history with greater than 3000 human cases reported between 2008 and 2009. In comparison, an average of 17 cases annually was reported in the Netherlands between 1978 and 2006 (Schimmer et al., 2009; van der Hoek et al., 2010). Living within 2 kilometers (1.25 miles) of a dairy goat farm with significant abortion issues was a risk factor for human cases of Q fever (Schimmer et al., 2010). An emergent strain of *C. burnetii* was identified by MLVA in the investigation of this outbreak (Roest et al., 2011) and has since been recognized in Belgium as well (Boarbi et al., 2014).

In 2011, an outbreak of Q fever in does on a farm in Washington State was reported. Goats from this farm had been purchased and transported to 21 farms in Washington, Montana and Oregon prior to the identification of *C. burnetii* on the original farm. Of 108 people tested by serology for exposure to *C. burnetii*, 20 were linked to at least one of the 17 farms of interest that participated in the broader investigation (Bjork & Anderson, 2011). Genotyping identified ST8 in 3 environmental samples and 3 animal isolates associated with this outbreak (Kersh et al., 2013). Research focusing on the farms involved in this outbreak also determined that human beings can act as fomites for *C. burnetii*, transferring the organism from barns into their

houses although the greatest concentrations of *C. burnetii* DNA were within 50m of the areas where parturition or abortion occurred (Kersh et al., 2013).

Outbreaks of Q fever have not been reported in Indiana. However, individual cases have been reported both to the Indiana State Department of Health (ISDH) and to the Indiana State Board of Animal Health (ISBOAH). The highest number of annual human cases, 4, was reported to the ISDH in 2005 (Beall et al., 2007). Reporting of suspected animal cases to the ISBOAH has also been infrequent with 2 positive cases documented in sheep in 2008, 1 positive case in a bovine in 2011, 2 positive bovine cases in 2013 and 5 positive cases in 2015 (1 canine and 4 caprine) (personal communication, IABOAH). The lack of reported cases of Q fever does not negate the importance of active research into *C. burnetii* in Indiana. Indeed, research into the prevalence of *C. burnetii* in reservoir species in Indiana can help to either support the appropriateness of passive surveillance efforts or indicate that active surveillance may be necessary.

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CHAPTER 3. PREVALENCE OF *COXIELLA BURNETII*, AGREEMENT BETWEEN DIAGNOSTIC METHODS AND RELATIONSHIPS WITH BREED PURPOSE IN INDIANA GOATS

3.1 Introduction

Coxiella burnetii is the causative agent of Q fever, a zoonotic disease with effects ranging from the absence of clinical signs to acute influenza-like symptoms to vegetative endocarditis in people (Raoult et al., 2005). First described among abattoir workers in Australia in 1935 (Derrick, 1937), human Q fever outbreaks continue to occur globally (Brouqui et al., 2004; Roest et al., 2011; Bjork et al., 2014; Eldin et al., 2014). Although it has been a reportable disease in the United States since 1999 (McQuiston et al., 2006), the clinical similarities of the acute form of Q fever to influenza likely lead to underdiagnosis and thus to underreporting.

Human exposure to *C. burnetii* can occur by several routes. Direct handling of placenta and other reproductive materials results in exposure to large numbers of *C. burnetii* (Arricau-Bouvery & Rodolakis, 2005). Wind transmission has been associated with human Q fever outbreaks (Tissot-Dupont et al., 1999; Tissot-Dupont et al., 2004; Schimmer et al., 2010). Both of these routes of transmission relate to inhalation of aerosolized *C. burnetii*. Outbreaks of Q fever have also been associated with the consumption of unpasteurized dairy products (Fishbein & Raoult, 1992; Signs et al.,

2012). Q fever is also considered to be a tick borne disease, but few cases of transmission of *C. burnetii* by ticks to people have been reported (Eklund et al., 1947). *C. burnetii* is presumed to be endemic in domestic ruminants (Norlander, 2000) and ubiquitous in the environment (Kersh et al.; 2010, Kersh et al., 2013), thus knowledge of the prevalence of *C. burnetii* in reservoir species can help public health agencies prepare for potential Q fever outbreaks.

Small ruminants are commonly associated with human *C. burnetii* infection and recent outbreaks in the Netherlands and the United States have been associated with goats (Roest et al., 2011; Bjork et al., 2014). In goats, late term abortion is the most common clinical manifestation, but infection with *C. burnetii* can have varied presentations, including the absence of symptoms (Arricau-Bouvery & Rodolakis, 2005). *C. burnetii* can be shed through the products of parturition, urine, feces and milk. The greatest number of bacteria are shed in the placenta (Roest et al., 2012), but other routes of shedding vary in importance by species (Rodolakis et al., 2007). Based on experimental studies, the primary route of *C. burnetii* shedding in goats after parturition or abortion is through milk although shedding in vaginal mucus and feces does occur (Rodolakis et al., 2007; Roest et al., 2012).

Given its ubiquity, qualifying a herd or location as *C. burnetii* free is difficult to impossible. However, a herd with no evidence of infection that has incorporated preventive measures into its management strategies may be considered low risk (NASPHV & NASAHO, 2013). Methods to determine low risk herd status have been defined, including serologic testing of the full herd, monitoring of bulk tank samples for

C. burnetii DNA by polymerase chain reaction (PCR) and testing of placentas in all aborted and select normal pregnancies by PCR (NASPHV & NASAHO, 2013). Despite recommendations for good biosecurity measures at the herd level and requirements for testing for exposure to *C. burnetii* prior to international shipment, there are limited recommendations for evaluating individual animals for infection with *C. burnetii* before or at the time of introduction to a herd or flock. Serologic testing of animals can indicate exposure to *C. burnetii* if a single sample is positive or active infection if seroconversion occurs between paired samples. Checking individual samples for the presence of organismal DNA indicates active infection if samples are positive, but shedding of *C. burnetii* can be intermittent (Arricau-Bouvery et al., 2003). Evaluation of the agreement between diagnostic methods in apparently healthy animals can provide a foundation for recommendations as to which test or combination of tests is most likely to detect an individual infected with *C. burnetii* prior to the introduction of new animals to a low risk herd or flock.

The first objective of this study was to estimate the prevalence of anti-*C. burnetii* antibodies and shedding of *C. burnetii* DNA among the general population of goats in Indiana at both the individual and herd levels. The second objective of this study was to evaluate agreement between serologic and molecular diagnostic methods. A third objective was to determine if a relationship between breed purpose and infection with *C. burnetii* could be identified.

3.2 Materials and methods

3.2.1 Inclusion criteria, and recruitment

The population of interest for this study was defined as goat does over 1 year of age and not pregnant at the time of sampling. Does were chosen as the focus of this study because the greatest number of *C. burnetii* organisms are shed in placenta and reproductive discharges at the time of abortion or normal parturition (Roest et al., 2012) and milk is the primary route of shedding in goats (Rodolakis et al., 2007). In order to include asymptomatic carriers of *C. burnetii*, there were no restrictions placed upon health status or reproductive history although information on reproductive history was collected. Sample size calculations to determine a target number of herds to recruit were based on an estimated seropositivity and shedding frequency of 50%, 95% confidence and a precision of +/-10%. In order to determine the number of does to test per herd to detect *C. burnetii* infection at the herd level, a within-herd seroprevalence of 40% and shedding frequency of 50% were assumed with a 95% confidence level defined. Based upon these definitions, sampling up to 10 does per herd would allow for detection of *C. burnetii* within the herd with 95% confidence in herds with up to 100 does.

Goat herds were identified through breed organization directories, internet searches and personal communication at shows between May of 2012 and August of 2014. Recruitment was through e-mail, telephone conversations and personal conversations, resulting in a convenience sample. The e-mail recruitment document can be found in Appendix A. Sampling also occurred between May of 2012 and August of

2014 at the show or at a farm visit based upon producer preference. In herds with more than 10 does that met the inclusion criteria, the producer selected those to be sampled. In herds with fewer than 10 does meeting the inclusion criteria, all were sampled.

3.2.2 Sample collection and storage

Blood and milk samples were collected at a single visit for each doe, as approved by the Purdue University Animal Care and Use Committee (PACUC protocol 1205000641). Blood samples were collected by jugular venipuncture into serum separator tubes by the investigators. Milk samples were collected by the owner or the investigators and normal hygienic procedures for that herd were followed. Sterile cotton-tipped plastic swabs were used by an investigator to collect samples of vaginal mucus. In order to ensure that the collected fecal samples represented a single doe, samples were collected from the rectum by an investigator unless the doe defecated at any point during sample collection, in which case the immediately voided sample was collected.

All samples were transported in coolers with ice or ice packs. Upon return to Purdue University, blood samples were centrifuged at 4,000 rpm for 10 minutes in order to separate the serum. After centrifugation, 1 to 2ml aliquots of serum were removed and placed in cryotubes for storage at -20^{o}C prior to testing. Samples were stored at -20^{o}C until DNA extraction was performed unless extraction was performed within 24 hours of sample collection. In this case, samples were kept refrigerated until extraction was performed and stored at -20^{o}C after extraction.

3.2.3 Laboratory analysis

Serum samples were tested for anti-*C. burnetii* antibodies using the Idexx Chekit Q-fever antibody test (trademark IDEXX Laboratories, Hoofddorp, the Netherlands) following the manufacturer's protocol. This enzyme-linked immunosorbent assay (ELISA) detects both phase I and phase II antibodies and is based upon an antigen derived from the Nine Mile strain of *C. burnetii*. In a study performed at the National Veterinary Service Laboratory (NVSL), the sensitivity of this test was estimated to be 97% and specificity was estimated to be 100% (Emery et al., 2014), which is a slightly lower sensitivity than documented by the manufacturer. Positive and negative controls provided with the kit and serum samples were diluted 1:400 in wash buffer and 100μl aliquots of this dilution were added to the wells of a microtiter plate coated with inactivated *C. burnetii* antigen and incubated at 37^{o}C for 60 minutes. Following incubation, the wells were washed in triplicate with 300μl of wash buffer, 100μl of anti-ruminant-IgG-PO conjugate labeled with horseradish peroxidase conjugate was added to each well and a second incubation at 37^{o}C for 60 minutes was performed. Following the second incubation, the wells were again washed in triplicate, 100μl of TMB substrate was added to each well and 15 minute incubation was performed at room temperature. Finally, 100μl of STOP solution was added to each well and the optical densities (OD) were read. Based upon the manufacturer's recommendations, a positive sample was defined as one that demonstrated an optical density greater than or equal to 40% of that of the positive controls run with the assay. A negative sample was

defined as one that demonstrated an OD less than 30% of that of the positive control after correction by subtraction of the OD of the negative control. Samples with OD between 30 and 40% of the positive control after correction by subtraction of the negative control were defined as indeterminate and retested.

A Qiagen DNeasy Blood and Tissue Kit (trademark Qiagen, Valencia, California) was used for the extraction of DNA from milk samples. After homogenization by vortex, 200μl of milk was combined with 180μl Buffer ATL and 20μl proteinase K, mixed by vortex and incubated for at least 3 hours at 55^{o}C. After this incubation, 400μl of Buffer AL was added to each sample and a 70^{o}C incubation was performed for 10 minutes after mixing by vortex. Ethanol (400μl) was added to the mix and the lysate was filtered through columns using Buffers AW1 and AW2. Buffer AE was added to the columns for a final elution volume of 200μl.

A Qiagen DNeasy Blood and Tissue Kit protocol for DNA extraction from buccal swabs was utilized for DNA extraction from the vaginal samples. Swabs were suspended in 400μl sterile PBS. After suspension, 20μl Proteinase K and 400μl Buffer AL were then added to each sample. Swabs and reagents were mixed by vortex, incubated at 56^{o}C for 10 minutes and briefly centrifuged prior to adding 400μl ethanol to the lysate. The lysate was filtered through columns and washed with buffers AW1 and AW2. Buffer AE (150μl) was added to the columns for the final elution.

A QIAamp DNA Stool Mini Kit (trademark Qiagen, Valencia, California) was utilized for DNA extraction from fecal samples. Individual sample aliquots of 200mg of feces

were used for each extraction. One ml of Inhibitex Buffer was added to each sample, vortexed to mix and incubated at 95^{o}C for 5 minutes prior to centrifugation. After centrifugation, 200µl of supernatant was added to 15µl Proteinase K and 200µl of Buffer AL was added. Samples were mixed by vortex and incubated at 70^{o}C for 10 minutes. After incubation, 200µl ethanol was added to the lysate, mixed by vortex and 600µl of the lysate was filtered through columns and washed with buffers AW1 and AW2. Buffer ATE (200µl) was used for the final elution. All DNA samples were stored at -20^{o}C prior to PCR testing.

The real time PCR targeted an 86 base pair section of the IS1111 transposon as defined by Panning (Panning et al., 2008). The forward primer CburF, 5'-GAT AGC CCG ATA AGC ATC AAC – 3' (Life Technologies, Carlsbad, CA, USA), reverse primer CburR, 5' – GCA TTC GTA TAT CCG GCA TC – 3' (Life Technologies, Carlsbad, CA, USA) and probe Cbur 5' – FAM – TCA TCA AGG CAC CAA T – MGBNFQ – 3' (Life Technologies, Carlsbad, CA, USA) were used (Panning et al., 2008). The reaction mixture consisted of 2.5µl of magnesium free 10x reaction buffer (Invitrogen, Life Technologies, Carlsbad, CA), 2.0µl of 50mM magnesium chloride (Invitrogen, Life Technologies, Carlsbad, CA), 0.5µl of 10mM dNTPs (Applied Biosystems, Warrington, UK), 2.0µl of CburF, 2.0µl of CburR, 0.5µl of Cbur probe, 0.1µl Platinum Taq polymerase (trademark Invitrogen, Life Technologies, Carlsbad, CA) and 10.15µl of nuclease free water plus 5 µl of DNA sample. A Stratagene Mx3000P qPCR system (trademark Agilent Technologies, Santa Clara, CA) was utilized to perform an amplification protocol of 95^{o}C for 10 minutes for strand

separation, followed by 45 repeated cycles of 95^{o}C for 15 seconds for strand separation and 60^{o}C for 30 seconds for annealing and amplification (Panning et al., 2008). Amplification data was collected in MxPro (trademark Agilent Technologies, Santa Clara, CA). Based upon a standard curve generated through use of a plasmid containing the 86 base pair target sequence the analytic sensitivity of this PCR was determined to be 100 copies of the IS1111 transposon (Bauer et al., 2015), having a CT of 36.5. Samples demonstrating cycle threshold (CT) values on the initial PCR were run in duplicate and those with average CTs less than or equal to 36.5 were considered positive for purposes of analysis. A negative control of nuclease free water and positive control of *C. burnetii* DNA extracted from cattle milk from a previous study (Bauer et al., 2015) were run with each PCR. Due to variation of copy numbers of the IS1111 transposon by strain of *C. burnetii* (Klee et al., 2006), the number of individual bacteria detectable by this assay was not determined.

3.2.4 Statistical analysis

Individual and herd level prevalence estimates and 95% confidence intervals (CI) were calculated in OpenEpi (Dean et al., 2014). The 95% confidence intervals were calculated using the Wilson score method. The sensitivity and specificity values reported in the NVSL study (97% and 100% respectively) (Emery et al., 2014) were used to calculate adjusted seroprevalence within the sample utilizing the formula: p(D+) = (Apparent prevalence + Specificity – 1)/(Sensitivity + Specificity – 1) (Dohoo et al., 2009). Individual level calculations for apparent prevalence were based upon the number of does positive for *C. burnetii* by the given diagnostic method divided by the total number

of does tested by that method. Herd level calculations were based upon the number of positive herds by the given diagnostic method divided by the total number of herds tested by that method. Prevalence estimates and 95% confidence intervals were calculated at both herd and individual levels for both seropositivity and shedding of *C. burnetii* DNA in all sample types tested by PCR.

In order to determine whether the serology and DNA shedding results were in agreement, Cohen's kappa (Cohen, 1960) was calculated in IBM SPSS Statistics for Windows version 22.0 (Released 2013 IBM Corp. Armonk, NY) for both individuals and herds. Point estimates for kappa as well as 95% confidence intervals are presented. Percent concordance between test results was also calculated.

A Chi-square test of association was performed in SPSS to evaluate the association between breed type and the binary laboratory test results for individual anti-*C. burnetii* antibodies and *C. burnetii* DNA shedding. The breed type was defined as dairy, meat, fiber or unknown, but due to small numbers of does in the fiber and unknown groups, only individuals classified as dairy or meat breeds were used in the analysis. Dairy breed was used as the comparison group for the calculation of odds ratios. A test of association was also run at the herd level to evaluate if there was an association between herds with meat breeds of goat present and seropositivity for *C. burnetii* or shedding of *C. burnetii* DNA.

3.3 Results

3.3.1 Sample

A total of 649 does representing 94 farms from 49 counties in Indiana were included in the study. Does ranged in age from 1 to 14 years with a median of 2 years. The majority of does sampled (n=414, 63.8%) were from dairy breeds, followed by meat breeds (n=223, 34.4%). Ten fiber breed does were included in the study. An unidentified breed group consisted of 2 individuals.

Farms included in this study were not necessarily limited to one breed of goat or even one breed type. Of farms limited to a single breed type, 48 had dairy breed goats only and 21 had meat breed goats only. A single farm had only fiber breed goats. Twenty-four farms had both dairy and meat breed goats present in the herd.

3.3.2 Prevalence estimates

Serum samples were collected from a total of 608 does representing 89 farms. Twenty-three of the samples tested by ELISA were positive for anti-*C. burnetii* antibodies. These samples came from 10 different farms. The estimated herd and individual seroprevalences for anti-*C. burnetii* antibodies are displayed in Table 3.1 and were 11.2% (95% CI: 6.2-19.5%) and 3.8% (95% CI: 2.5-5.6%), respectively. Utilizing the sensitivity and specificity values calculated at the NVSL (Emery et al., 2014) to calculate real prevalence did not result in any changes in the seroprevalence estimates.

DNA samples from at least one of the three sources (milk, vaginal swabs or feces) were tested for the IS1111 transposon by real time PCR for 649 does, representing all 94 of the farms. A total of 49 does were positive for *C. burnetii* DNA by at least 1 route of

shedding. The positive samples came from 19 different farms. The estimated individual and herd level prevalences for shedding *C. burnetii* DNA are displayed in Table 3.1 and were 7.5% (95% CI: 5.7-9.8%) and 20.2% (95% CI: 13.3-29.4%) respectively.

Table 3.1 also includes prevalence estimates based upon the source of the DNA sample. Milk samples were collected from 387 does representing 83 farms. Nine of the individual milk samples from 6 farms were positive for *C. burnetii* DNA. The estimated prevalence of shedding *C. burnetii* in milk by individual does was 2.3% (95% CI: 1.2-4.4%). The estimated herd level prevalence of *C. burnetii* infection based on milk samples was 7.2% (95% CI: 3.3-14.9%).

Swab samples of vaginal mucus were collected from 632 does representing 92 farms. Forty of these samples from 14 farms were positive for *C. burnetii* DNA. The estimated prevalence of *C. burnetii* shedding by individual does in Indiana in reproductive discharges was 6.3% (95% CI: 4.7-8.5%). The estimated herd level prevalence for *C. burnetii* infection based on sampling reproductive discharges was 15.2% (95% CI: 9.3-23.9%).

Fecal samples were collected from 574 individual does representing 92 farms. Only a single fecal sample tested positive for *C. burnetii* by PCR. The estimated prevalence of *C. burnetii* based on testing fecal samples was 0.2% (95%CI: 0.03-1.0%). At the herd level, the estimated prevalence of infection with *C. burnetii* based upon fecal samples was 1.1% (95% CI: 0.2-5.9%).

3.3.3 Agreement between diagnostic samples

Table 3.2 details the relationship between serology and overall PCR results. Both serum and DNA samples were tested in 608 does from 89 farms. Six does from 3 farms were positive for *C. burnetii* by both ELISA and PCR. Five-hundred-forty-three does from 63 farms were negative for *C. burnetii* by both ELISA and PCR. In general, the agreement between ELISA and PCR was poor both at the individual (kappa=0.12, 95%CI: 0.005-0.24) and farm (kappa=0.07, 95%CI: -0.14, 0.28) levels, but percent concordance, which takes into account agreement between negative test results, was high at both levels (90.3% and 70.8% respectively).

When the specific types of samples used as DNA sources were evaluated for agreement with serologic results (Table 3.3 and Table 3.4), agreement was consistently poor. The agreement between ELISA and PCR detection of *C. burnetii* DNA in milk samples was 0.04 (95% CI: -0.10-0.18) for the individual does tested and 0.17 (95%CI: -0.12 – 0.47) at the herd level. Percent concordance was 93.0% for individuals and 85.0% at the herd level. The measure of agreement between ELISA and PCR of samples collected by vaginal swab was 0.16 (95% CI: 0.02-0.29) and percent concordance was 91.9% for the individual does tested. At the herd level, kappa was 0.04 (95%CI: -0.18-0.26) and percent concordance was 77.5%. The measure of agreement between ELISA and PCR of fecal samples was -0.003 (95% CI: -0.009, 0.003) and percent concordance was 96.3% for individual does. At the herd level, the kappa coefficient was -0.02 (95% CI: -0.06-0.02) and percent concordance was 87.5%.

3.3.4 Comparing meat and dairy breeds

The majority of the 598 does serosampled and included in breed analysis were from dairy breeds (64.2%, Table 3.5). Meat breeds provided 35.8% of the samples. Twenty-two of the 89 farms providing serum samples had both meat and dairy breeds present in their herds (24.7%). Twenty-one farms (23.6%) had only meat breeds in their herds and 45 farms (50.6%) had only dairy breeds. Seventeen dairy does were seropositive for anti-*C. burnetii* antibodies, a seroprevalence within the dairy breeds of 4.4% (95% CI: 2.8-6.9%). Six meat does were positive for anti-*C. burnetii* antibodies, a seroprevalence within the meat breeds of 2.8% (95% CI: 1.3-6.0%). No statistically significant difference in seroprevalence between dairy and meat breeds was detected by the cross-tab analysis, although there was a decreased odds of detecting anti-*C. burnetii* antibodies in meat breed does when compared to dairy breed does (OR=0.62, 95% CI: 0.24–1.60, p=0.32). There was no practical or statistically significant difference in the odds of a farm testing positive for anti-*C. burnetii* antibodies based upon the presence of meat breed goats in the herd (OR=1.08, 95% CI: 0.29–4.02, p=0.91).

The majority of the 637 does providing DNA samples and included in breed analysis were from dairy breeds (65.0%, Table 3.5). Meat breeds provided 35.0% of the samples. Twenty-three of the 93 farms providing serum samples had both meat and dairy breeds present in their herds (24.7%). Twenty-one farms (22.6%) had only meat breeds in their herds and 49 farms (52.7%) had only dairy breeds. Forty-one of the dairy breed does tested positive for *C. burnetii* DNA. Prevalence of shedding *C. burnetii* within the dairy does in this study was 9.9% (7.4-13.1%). Eight of the meat breed does tested

positive for *C. burnetii* DNA. Prevalence within the meat breed does in this study was 3.6% (1.8-6.9%). Overall, a meat breed doe had a reduction in the odds of testing positive for *C. burnetii* in a DNA sample of 3.3 folds as compared to a dairy breed doe (OR=0.34, 95% CI: 0.16–0.73, p=0.006).

Tables 3.6 and 3.7 detail the relationships between meat and dairy breed does and testing positive for *C. burnetii* DNA in milk and vaginal swab samples at the individual and herd levels. It is of note that meat breed does were 8.7 times more likely to test positive for *C. burnetii* DNA in a milk sample than dairy breed does (OR=8.69, 95% CI: 1.81-55.24, p=0.0049) , but had an 11.1 fold reduction in the odds of testing positive in a vaginal swab sample (OR=0.09, 95% CI: 0.02-0.37, p<0.001). There was no practical or statistically significant difference in the odds of a farm testing positive for *C. burnetii* DNA based upon the presence of meat breed goats in the herd (OR=0.79, 95% CI: 0.28–2.18, p=0.65). The presence of meat breeds in the herd had no statistically significant effect on the detection of *C. burnetii* DNA in either milk or vaginal mucus samples.

3.4 Discussion

Estimations of *C. burnetii* prevalence vary depending the sample source and type of testing. The estimated seroprevalence of 3.8% in Indiana determined in this study is somewhat lower than the seroprevalence findings of 8.0% reported in Washington State (Sondgeroth et al., 2013) and somewhat higher than 1.2% reported in Boer goats in Missouri (Baker & Pithua, 2014). The overall 7.5% prevalence of shedding of *C. burnetii* DNA in this study is lower than that determined in other studies (Arricau-Bouvery et al., 2003). In a study comparing shedding routes in does from farms under outbreak

conditions, milk samples tested positive for *C. burnetii* DNA in 38% of does that had experienced abortion and 31% of does with normal parturition (Rousset et al., 2009).

Lower prevalence estimates in this study may reflect either the choice to sample outside of an ongoing outbreak, or the timing of sampling in relation to parturition. After experimental infection by subcutaneous injection, milk shedding was found to continue for 52 days after abortion (Arricau-Bouvery et al., 2003). Sampling dates in this study ranged from less than 1 month to 36 months after the most recent freshening and the period for shedding of *C. burnetii* DNA may have been missed in those individuals that had freshened several months prior to sampling.

Based on the use of Cohen's kappa, seropositivity for *C. burnetii* has poor agreement with shedding of the bacteria as determined by identification of the IS1111 transposon at both the individual and herd levels. Although specifically developed for nominal scales (Cohen, 1960), a kappa coefficient was chosen to evaluate agreement between test results in this study because all results were defined as binary (positive or negative for evidence of *C. burnetii*) and thus no linear relationship could be assumed. A weakness of the kappa coefficient is its dependence upon prevalence (Byrt et al., 1993). Percent concordances between ELISA and overall PCR results in this study were 90.3% at the individual level and 70.8% at the herd level indicating that the majority of the paired samples were in agreement as negative results. The discrepancy between agreement and concordance is in part due to the low prevalence of *C. burnetii* in the does and herds in this study as the majority of the concordant pairs at both levels are negative results (Table 3.2). The low seroprevalence (3.8% at the individual level and

11.2% at the herd level) and DNA shedding prevalence values (7.5% at the individual level and 20.2% at the herd level) in this study contribute to a low kappa value.

Choosing to focus on individuals with a history of recent reproductive disease may have helped to address the effect of low prevalence on the kappa statistics. Reproductive history did not play a defined role in sample selection in this study, although since this was a convenience sample selection bias is certainly a concern in interpretation of the results. Animals living on farms where reproductive events have occurred would be more likely to have been exposed to, and exhibit titers against, *C. burnetii* as compared to animals on farms where reproductive events have not occurred. However, it is likely that the kappa statistic would indicate a poor level of agreement even if the prevalence estimates in this study were higher. The lack of agreement between molecular and serologic tests for *C. burnetii* previously documented in the contexts of outbreaks of Q fever and in experimental research, supports this conclusion. Eighteen of 72 goats identified as shedding *C. burnetii* DNA during a Q fever outbreak were negative for anti-*C. burnetii* antibodies by the same ELISA used in this study (Rousset et al., 2009). After experimental infection, fewer than half of the infected does (6/19) had detectable anti-*C. burnetii* antibodies at the time of abortion, regardless of the dose of *C. burnetii* administered (Arricau-Bouvery et al., 2003).

No statistically significant difference was noted between seroprevalence of anti-*C. burnetii* antibodies in meat breeds of goat as compared to dairy breeds. Although an odds ratio of 0.6 was calculated when comparing meat breeds to dairy breeds by exact logistic regression, wide confidence intervals indicate that the sample size is too small to

draw a clear conclusion about this relationship. There was a statistically significant difference noted in the prevalence of *C. burnetii* DNA in milk from meat breeds of goats as compared to dairy breeds and meat breed does were 8.7 times more likely to be shedding *C. burnetii* in milk samples. This finding may be related to clustering of animals as 4 of these does were from the same farm.

A unique feature of this study sample is that farms did not necessarily have a single breed, or even only dairy or meat breeds, present in the herd. This created an opportunity to evaluate the effect of mixed purpose herds on the odds of detecting infection with *C. burnetii*. In this study, farms that included meat breeds of goat in their herds had no statistically significant increase in the odds of being positive for either anti-*C. burnetii* antibodies or shedding of *C. burnetii* DNA than those farms that did not have goats from meat breeds in the herd.

Breed purpose may be a surrogate for either management factors that vary between meat and dairy operations or for physiologic or immune differences between meat and dairy breeds. At least one seroprevalence study has been performed focusing on a single breed of goat, the Boer goat (Baker & Pithua, 2014). However, breed specific studies evaluating shedding of *C. burnetii* DNA have not been performed. Studies focusing on breed classifications or specific breeds, particularly if does that had experienced reproductive events were the group of interest, would help to elucidate whether the predisposition to *C. burnetii* shedding in milk identified here is a generalizable finding, and if so can be associated with husbandry rather than the breeds themselves.

In conclusion, seroprevalence of *C. burnetii* in goats in Indiana and the prevalence of does shedding *C. burnetii* DNA are both low in Indiana. However, 20% of the herds included in this study had at least 1 doe shedding *C. burnetii* DNA at the time of sampling. This represents a potential for exposure to people interacting with these herds. In this study, there was a greater proportion of *C. burnetii* DNA shed in milk samples from meat breed does as compared to dairy breeds, which may reflect the clustering effect of farm, differences in husbandry between goats used for different purposes or intrinsic factors related to breed. Finally, while not precluding serology as a screening method for exposure to *C. burnetii*, lack of agreement between results of ELISA and PCR at both the individual and herd levels indicates that a single serum sample is not an appropriate choice for identification of active *C. burnetii* infection in individual does prior to introduction to a low risk herd. Indeed, the presence of the lack of agreement indicates that exclusion of individual animals from a herd based solely on seropositivity will not prevent entry of *C. burnetii* into that herd.

3.5 Bibliography

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Table 3-1. Estimated individual and herd level prevalence by sample type

Sample	Number of does	Number of positive does	Estimated Prevalence	95% CI	Number of herds tested	Number of positive herds	Estimated herd prevalence	95% CI
Serum	608	23	3.8%	2.5 – 5.6%	89	10	11.2%	6.2 – 19.5%
DNA	649	49	7.5%	5.7 – 9.8%	94	19	20.2%	13.3 – 29.4%
Milk	387	9	2.3%	1.2-4.4%	83	6	7.2%	3.3-14.9%
Vaginal	632	40	6.3%	4.6-8.5%	92	14	15.2%	9.3-22.9%
Feces	574	1	0.2%	0.03-1.0%	92	1	1.1%	0.2-5.9%

Table 3-2. Concordant and discordant results between ELISA and PCR samples at the individual and herd levels.

	Test			PCR	
			Positive	Negative	Total
Individual		Positive	6	17	23
		Negative	42	543	585
	ELISA	Total	48	560	608
Herd		Positive	3	7	10
		Negative	16	63	79
		Total	19	70	89

Table 3-3. Concordant and discordant results between ELISA and PCR at the individual level when compared by the source of DNA

Test						PCR				
			Milk			Swab			Feces	
		Positive	Negative	Total	Positive	Negative	Total	Positive	Negative	Total
ELISA	Positive	1	17	18	6	16	22	0	20	20
	Negative	8	330	338	33	549	582	1	528	529
	Total	9	347	356	39	565	604	1	548	549

Table 3-4. Concordant and discordant results between ELISA and PCR at the herd level when compared by the source of DNA

Test						PCR				
			Milk			Swab			Feces	
		Positive	Negative	Total	Positive	Negative	Total	Positive	Negative	Total
ELISA	Positive	2	8	10	2	8	10	0	10	10
	Negative	4	66	70	12	67	79	1	77	78
	Total	6	74	80	14	75	89	1	87	88

Table 3-5. ELISA and PCR results for meat breeds as compared to dairy breeds at the individual and herd level

Individual Breed type		ELISA			PCR	
	Positive	Negative	Total	Positive	Negative	Total
Meat	6	208	214	8	215	223
Milk	17	367	384	41	373	414
Total	23	575	598	49	588	637
Herd type						
Meat present	5	38	43	8	36	44
Dairy only	5	40	45	11	38	49
Total	10	78	88	19	74	93

Table 3-6. Comparing the proportions of individual meat and dairy breed does testing positive for *C. burnetii* DNA collected from milk and reproductive sources

Breed type		Milk			Reproductive	
	Positive	Negative	Total	Positive	Negative	Total
Meat	6	70	76	2	218	220
Dairy	3	304	307	38	362	400
Total	9	374	383	40	580	620

Table 3-7. Comparing the proportions of herds containing meat breed does and dairy breed does only testing positive for C. burnetii DNA collected from milk and reproductive sources

		Milk			Reproductive	
	Positive	Negative	Total	Positive	Negative	Total
Farm type						
Meat present	3	33	36	4	40	44
Milk only	3	46	49	10	38	48
Total	6	79	85	14	74	92

CHAPTER 4. GEOGRAPHIC DISTRIBUTION OF *COXIELLA BURNETII* IN INDIANA GOATS

4.1 Introduction

Coxiella burnetii is an obligate intracellular bacterium distantly related to the *Ricketsiales* and *Legionellaceae* (Drancourt & Raoult, 2005). Infection with this zoonotic agent is often inapparent in both human and animal hosts, but the clinical disease, Q fever, can be dramatic in its manifestations. In humans the acute form of Q fever results in influenza-like symptoms that have been reported to last up to 57 days without treatment (Parker et al., 2006). Pneumonia, hepatitis and pregnancy loss can also occur as complications of acute Q fever. The chronic form of Q fever most commonly manifests as endocarditis, but chronic fatigue syndrome and recurrent pregnancy loss can also occur. Individuals with immunosuppressive conditions or a history of cardiovascular disease are at a greater risk of developing chronic Q fever (Raoult et al., 2005).

Q fever was classified as a reportable disease in people in the United States in 1999. The number of cases reported annually in the United States between 2000 and 2007 ranged from 17 to 167 and demonstrated an increasing trend (CDC, 2013). This trend may be due to an increasing incidence of Q fever or it may be that designation as reportable increased physician awareness of the disease. However, given the clinical similarities

between acute Q fever and influenza, there is still likely to be underdiagnosis and underreporting. In Indiana, the number of annually reported cases of Q fever ranged from 0 to 4 between 2000 and 2007. The peak number of cases, 4, was reported in 2005 (Beall et al., 2002; Beall et al., 2004; Beall et al., 2006a; Beall et al., 2006b; Beall et al., 2007a; Beall et al., 2007b; Beall et al., 2007c; Staggs et al., 2009).

Domestic ruminants are the main reservoir hosts associated with human Q fever. In recent years goats have been associated with several outbreaks of Q fever in people. The largest historically documented outbreak of Q fever occurred in the Netherlands and was associated with dairy goats (Roest et al., 2011). The first American outbreak involving multiple states was also associated with goats (Bjork et al., 2014). As with human infection with *C. burnetii*, there are often no clinical signs of infection in goats. The most common clinical sign in this species is late term abortion, which is similar to several other pathogens including *Toxoplasma gondii* and *Leptospira interrogans* (Merck, 2005). Large numbers of *C. burnetii* are shed into the environment at the time of abortion and normal parturition. This, along with an infectious dose of *C. burnetii* of 1 to 10 organisms and an aerosol route of transmission, (Oyston & Davies, 2011) poses a great risk of infection for people assisting with and cleaning after parturition. Shedding of *C. burnetii* also occurs through milk and through fecal material (Rodolakis et al, 2007). Between 2008 and 2015, 26 animals tested for *C. burnetii* were reported to the Indiana State Board of Animal Health (personal communication). Ten of these animals were confirmed positive for *C. burnetii* either by complement fixation or polymerase chain

reaction (PCR) testing. Eight of the animals tested were goats and 4 of these were confirmed positive for infection with *C. burnetii* by PCR.

The purpose of this study was to evaluate the geographic distribution of *C. burnetii* infection of goats in Indiana. A secondary objective of this study was to identify and evaluate the geographic distribution of the genetic strains or sequence types (ST) of *C. burnetii* present in goats in Indiana. Although Q fever is a rare diagnosis in people in Indiana, identifying high risk regions of the state can help to plan intervention and surveillance measures aimed to promote early detection and control of *C. burnetii* outbreaks.

4.2 Materials and methods

A convenience sample was collected for this study. Farms with goats were identified using breed organization directories, internet searches and personal communications at shows. Recruitment of farms was performed at shows or through e-mail and telephone conversations. Producers that agreed to participate provided informed consent for sample collection from does over 1 year of age and not pregnant at the time of sampling as approved by the Purdue University Animal Care and Use Committee (protocol 1205000641). The producers also provided informed consent to participate in surveys about their farms, husbandry procedures and histories of the individual goats sampled. These surveys were approved by the Purdue University Institutional Review Board and included questions about the zip code and county of the farm's location in addition to history of showing and methods of breeding. Farms and animals were identified by a numeric code rather than name to protect confidentiality.

Participants were advised that they could choose to decline certain sampling procedures, refuse to answer certain questions on the surveys and withdraw from participation in the study at any time.

Geographic regions of Indiana were defined based upon zip codes, public health preparedness districts (PHPD) and regions (see Figure 4.1 for PHPD and region boundaries). The number of goat farms and individual goats in each PHPD and region was calculated upon the National Agricultural Statistics Service's Census of Agriculture for 2012 (USDA, 2012). The percentage of goat farms per PHPD and region were calculated in order to evaluate the representativeness of the sample as compared to the known distribution of goats in Indiana in 2012. Maps of the geographic regions as well as the geographic distributions of goats and farms in 2012 and the locations of the goats and farms sampled at the zip code level were generated using the ArcMap function in ArcGIS10.3 (Copyright © Esri. All rights reserved).

4.2.1 Diagnostic samples

After sample collection, samples were transported to the laboratory at Purdue University on ice. Serum was separated by centrifugation at 4,000 rpm for 10 minutes and 1 to 2 ml aliquots were removed and stored at -20^{o}C. The presence of anti-*C. burnetii* antibodies in each sample was identified by use of the commercially available Chekit-Q fever enzyme-linked immunosorbent assay (ELISA) (trademark IDEXX Laboratories, Hoofddorp, the Netherlands). This test detects both phase I and phase II antibody responses to *C. burnetii* infection. Analyses were performed following the manufacturer's instructions. Paired negative and positive controls provided by the

manufacturer were tested with each group of samples. Positive samples demonstrated an optical density greater than or equal to 40% of that of the positive controls run with the assay. A negative sample was defined as one that demonstrated an OD less than 30% of that of the positive control after correction by subtraction of the OD of the negative control. Samples with OD between 30 and 40% of the positive control after correction by subtraction of the negative control were defined as indeterminate and retested.

Samples of milk, vaginal mucus and feces were collected from each individual doe for DNA extraction and testing for the presence of *C. burnetii* by real time polymerase chain reaction (PCR). All samples were transported from the farm or show to Purdue University on ice and stored at -20^{o}C unless DNA extraction was performed within 24 hours of sample collection. In the latter circumstance, samples were stored under refrigeration until extraction could be performed. After homogenization using a vortex, 200µl of milk was utilized as the source for DNA extraction by column with a Qiagen DNeasy Blood and Tissue Kit (trademark Qiagen, Valencia, California) following a previously reported protocol (Bauer et al., 2015).

The Qiagen DNeasy Blood and Tissue Kit™ was also utilized for extraction of DNA from the sterile cotton-tipped swabs used to collect vaginal discharges. The manufacturer's protocol for DNA extraction from cheek swabs was followed. Swabs were suspended in 400µl sterile PBS, 20µl Proteinase K and 400µl Buffer AL were then added to each sample. Swabs were mixed by vortex, incubated at 56^{o}C for 10 minutes and briefly centrifuged prior to adding 400µl ethanol to the lysate. The lysate was

filtered through columns and washed with buffers AW1 and AW2. Buffer AE was added to the columns for a final elution volume of 150μl.

A QIAmp DNA Stool Mini Kit™ (Qiagen, Valencia, California) was utilized to perform DNA extraction from the fecal samples following the manufacturer's protocol with the adjustment of temperature to 95^{o}C for the initial incubation step. In brief, an initial volume of 200mg of feces was suspended in 1ml of Inhibitex Buffer, mixed by vortex and incubated at 95^{o}C for 5 minutes prior to centrifugation. After centrifugation, 200μl of the resulting supernatant was added to 15μl Proteinase K followed by 200μl of Buffer AL. Samples were mixed by vortex and incubated at 70^{o}C for 10 minutes. Following this final incubation, 200μl ethanol was added to the lysate, mixed by vortex and 600μl was filtered through columns and washed with buffers AW1 and AW2. Buffer ATE was used for the final elution, producing a final volume of 200μl.

Real time PCR was utilized to amplify an 86 base pair section of the IS1111 transposon of *C. burnetii* (Panning et al., 2008; Bauer et al., 2015). Samples demonstrating an average cycle threshold value of 36.5 or less on repeated amplifications were defined as positive for the presence of *C. burnetii* DNA. This cut-off value was determined based upon evaluation of a standard curve developed for the target DNA sequence through use of a plasmid created by the pGEM –T vector system (trademark Promega Corporation, Madison, Wisconsin, USA). For the purposes of the current study, individual does that tested positive either for anti-*C. burnetii* antibodies or for *C. burnetii* DNA in at least 1 sample were defined as positive individuals.

4.2.2 Sequence typing

DNA from selected vaginal swab samples was submitted to the Center for Microbial Genetics and Genomics at Northern Arizona University for genotyping utilizing single nucleotide polymorphism (SNP) analysis to identify the multispacer sequence type (MST) (Glazunova et al, 2005). TaqMan assays for SNPs at 2 loci on the IS1111 transposon were performed (Hornstra et al, 2011). The loci tested, 51bp67 and 22bp118, were chosen to target ST8, the most common *C. burnetii* ST identified in samples from goats in the United States (Pearson et al, 2014). The reaction mix for each sample consisted of 5μl 2x TaqMan Universal PCR Master Mix (trademark Life Technologies, CA, USA), 0.45 μl of each primer, 0.1μl of each probe and 2.9μl of sterile nuclease-free water to which 1μl of DNA was added for a total reaction volume of 10μl (Hornstra et al., 2011). An applied Biosystems 7900HT Fast real-time PCR system was utilized for reaction conditions of: 50^{o}C for 2 minutes, 95^{o}C for 10 minutes, 45 cycles of 95^{o}C for 15 seconds and 60^{o}C for 1 minute and Sequence Detection System v2.4 software (trademark Life Technologies, CA, USA) was utilized to recognize and record the SNPs (Bauer et al., 2015).

4.2.3 Statistical analysis

In order to evaluate how well the study sample represented the true geographic distribution of goats in Indiana, the percentage of total does sampled from a given PHPD and region of Indiana were compared with the percentage of total does reported in the 2012 National Census of Agriculture (USDA 2012) for the same PHPD and region of the

state utilizing a chi-square goodness of fit test in SPSS (SPSS Statistics for Windows, Version 22.0 Armonk, NY: IBM Corp Released 2013). The expected number of sampled goats for each region or PHPD were calculated based upon the percent of the total goats in Indiana present in that PHPD or region. Chi-square tests of association were also performed to evaluate deviations between the expected prevalence of *C. burnetii*, calculated based on the overall estimated prevalence for the state of Indiana, in a given region or PHPD and the observed prevalence of *C. burnetii* at the individual and farm levels. Where statistically significant differences were detected by the chi-square tests of association, odds ratios comparing each region to a selected comparison region and each PHPD to a selected PHPD were calculated in SPSS.

4.3 Results

4.3.1 Geographic associations

Based on the information collected in the 2012 Census of Agriculture, there were 38632 goats on 2883 premises in the state of Indiana (USDA, 2012). As illustrated in Figure 4.2, the number of goats reported per county ranged from 17 in Union County to 2618 in Elkhart County. The median number of goats per county was 351.5. The number of premises owning goats per county ranged from 5 farms in Union and Marion Counties to 121 farms in LaGrange County. The median number of farms per county was 27.5. Samples in this study were collected between May of 2012 and August of 2014 from 649 goats representing 94 farms from 49 counties in Indiana. Each of the 3 regions and 10 PHPDs in Indiana was represented in this sample (Table 4.1). Based on the Chi-square goodness of fit analysis, there was a statistically significant difference between the

spatial distribution of the total number of goats in Indiana and the study sample at both the regional and the PHPD levels ($p<0.001$ for both levels). The representation of PHPDs 2, 3, and 9 differed by at least 5 percentage points between the sample and the overall population of goats in Indiana. At the regional level, the percentage of goats sampled from the Southern region differed from the population percentage by 7 percent.

Table 4.2 details the prevalence estimates for individual does by region and PHPD. At the state level, 10.2% of the does (n=66) tested positive for *C. burnetii* by either serology or PCR. Regionally, the Southern region had the highest prevalence of does testing positive for infection with *C. burnetii* (13.5%, n=22). There was no statistically significant difference detected between regions in the prevalence of infection with *C. burnetii* in individual does ($p=0.20$). At the PHPD level, PHPD 9 had the greatest prevalence of *C. burnetii* in the sampled does (20.3%, n=16) while PHPD 7 did not have any does test positive for *C. burnetii*. There was a statistically significant difference detected between PHPDs in the prevalence of *C. burnetii* infection in individual does ($p=0.042$).

Table 4.3 details the farm level prevalence estimates by region and PHPD. At the state level, 28.7% of the farms (n=27) had at least 1 doe that tested positive for *C. burnetii* by either serology or PCR. The Southern region also had the largest proportion of positive farms (34.8%, n=8). There was no statistically significant difference detected between regions in the prevalence of infection with *C. burnetii* at the farm level ($p=0.75$). At the farm level, PHPD 9 also had the highest prevalence of *C. burnetii* in the sampled

farms (44.4%, n=4). There was no significant difference detected between PHPDs in the prevalence of *C. burnetii* at the farm level (p=0.83).

Odds ratios were calculated for *C. burnetii* infection in individual does at the PHPD scale. PHPD 1 was chosen as the comparison district and results are shown in Table 4.4. There was no statistically significant difference in the odds of a doe testing positive for *C. burnetii* detected among the PHPDs. However, there was a pronounced increase in the odds of a doe testing positive for *C. burnetii* in PHPD 9 as compared to PHPD 1 (OR=4.09, 95%CI: 0.89-19.20).

4.3.2 Genotyping

Although 162 DNA samples (156 vaginal swab and 6 milk) were submitted for genotyping, a sequence type was identified for only 3 of the samples. These were identified as ST8 through the presence of the derived nucleotide C at Cox51bp67. An additional sample could not be ruled out from being ST8 based upon the amplification of the derived allele at site 22bp118, but could not be confirmed as ST8. The 4 samples were from 3 different farms. There was no apparent geographic relationship between the farms as 1 was located in Steuben County (PHPD 3, Northern region), 1 in Henry County (PHPD 6, Central region) and 1 in Decatur County (PHPD 9, Southern region). The small number of samples successfully genotyped precluded further statistical analysis.

4.4 Discussion

In general, outbreaks of Q fever occur at local or regional scales rather than at the national level or international level (Schimmer et al., 2008; Signs et al., 2012; Bjork et al., 2014). Moreover, infection with *C. burnetii* is not always expressed as clinical Q fever in the human or ruminant hosts. Identifying a predisposition to infection with *C. burnetii* in the host species in a particular geographic location can facilitate resource allocation for education and interventions as well as help to focus surveillance strategies for both human and animal populations. In this study, a trend toward increased odds of infection with *C. burnetii* was identified for does from PHPD 9, which encompasses 12 counties in the southeastern part of Indiana. Three of the counties with the largest populations of goats in 2012 (Harrison, Jefferson and Ripley counties) are located within PHPD 9. This study included 2 farms from Harrison County, both of which had goats that tested positive for *C. burnetii* by PCR, but neither Jefferson County nor Ripley County was represented. The other counties PHPD 9 with positive goats, Decatur and Scott Counties, were each represented by a single farm with positive does.

Limitations in this study derive in part from the use of a convenience sample. Relying on voluntary participation can affect both the sample size and how well the sample reflects the population of interest. Small sample size is an important limitation of this study. Approximately half of the counties sampled (n=24) are represented by a single herd of goats. This may not be as problematic for counties with a small number of goats/farms such as Perry county (165 goats in 2012), but is of concern for counties with a large goat population such as Elkhart and Daviess counties (2618 and 901 goats

respectively). The presence of *C. burnetii* in counties with only a single farm sampled could have been missed, resulting in underestimation of prevalence for that PHPD. This may be the case for PHPD 7, although 5 farms from that PHPD were sampled. Alternately, if the herd tested was positive for the presence of *C. burnetii,* the prevalence for the PHPD would have been overestimated. The limited number of counties with more than 1 herd tested also precluded the estimation of prevalence at the county level. All PHPDs had at least 5 herds tested which allowed statistical evaluation, but limited the precision of prevalence and odds ratio estimates as illustrated in the wide confidence intervals in Tables 2, 3, and 4. The lack of statistical significance for the increased odds of a doe testing positive for *C. burnetii* in PHPD 9 may also be related to small sample size.

An additional challenge of a convenience sample is the introduction of sampling bias. In this study, the sample did not accurately reflect the spatial distribution of the study population, introducing bias into the results. A larger sample size would not have necessarily corrected this bias, but combining a larger sample size with the goal of recruiting farms to the study in numbers proportionate to the number of farms present in each county, a sampling proportionate to size strategy, may have helped. For example La Grange County had 121 farms which owner goats in 2012, 4.2% of the total farms with goats in Indiana. In a study with a target sample size of 200 farms, 8 of those farms would be recruited from LaGrange County. Convenience sampling would still play a role in recruitment within the county, but this recruitment method would result in a more geographically representative sample.

Goats in this sample were not necessarily geographically restricted, resulting in a third limitation of this study. Of the 94 farms included in the study, 78 (83.0%) owned goats for the purpose of showing and 45 (47.9%) bred their does to bucks that they did not own by natural insemination. Of these 45 farms, 37 (82.2%) transported the does to the premises where the bucks were housed for breeding. Goats do not tend to stay in one location and this should be accounted for in future studies.

SNP analysis to identify the STs of *C. burnetii* circulating within Indiana was successful in only 3 samples although a probable ST was identified in an additional sample. This lack of success was in part due to contamination of many of the vaginal swab samples by the plasmid designed to evaluate the analytic sensitivity of the real-time PCR utilized in this study. A real-time PCR designed to target the vector portion of the plasmid genome was used to determine that the positive vaginal samples reported as positive herein were free of plasmid contamination. In addition to the challenge of contamination, a low amount of target DNA for the SNP analyses in the submitted samples may also be contributing the low number of samples successfully genotyped. The PCR utilized in this study targets the IS1111 transposon. This region of the *C. burnetii* genome is present in multiple copies, but there is variation in the number of copies present by strain (Klee et al., 2006). The target genes utilized for SNP analysis are single copy and thus detection of high numbers of the IS1111 transposon in a DNA sample does not guarantee that there are many copies of the SNP targets present in the same sample. This was a similar challenge when sequence typing of *C. burnetii* samples from bulk tank milk samples was undertaken (Bauer et al., 2015). Bulk tank milk samples

are contributed to by multiple individuals and thus are likely to contain larger quantities of bacteria than the samples in the current study, which came from individual goats. The samples where a sequence type was successfully identified are consistent with a previous study documenting ST8 as the most common strain of *C. burnetii* in goats in the United States (Pearson et al., 2014).

C. burnetii was identified in goats from all regions of Indiana and all PHPDs with the exception of PHPD 7. There was a predisposition for does in the southeastern region of the state, PHPD 9, to test positive for *C. burnetii*. This region would be a good choice for research focused at a finer geographic scale, particularly investigations of patterns of transmission between goats and other species. PHPD 9 would also be a good choice for pilot testing educational measures and preventive interventions focusing upon producers, veterinarians and physicians.

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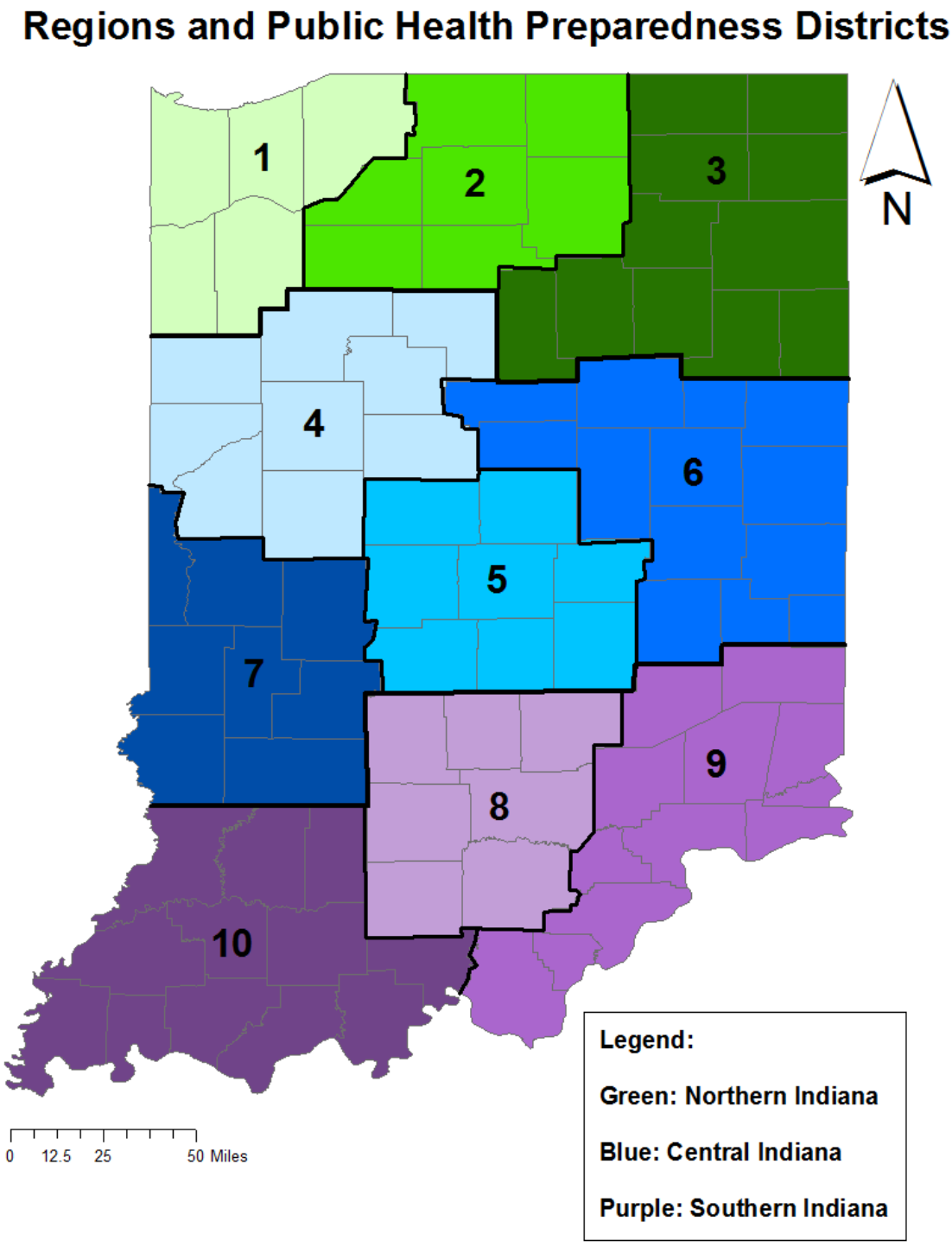

Figure 4-1. Public health preparedness district and regional boundaries in Indiana. Public Health Preparedness districts are identified by number while regions are identified by color.

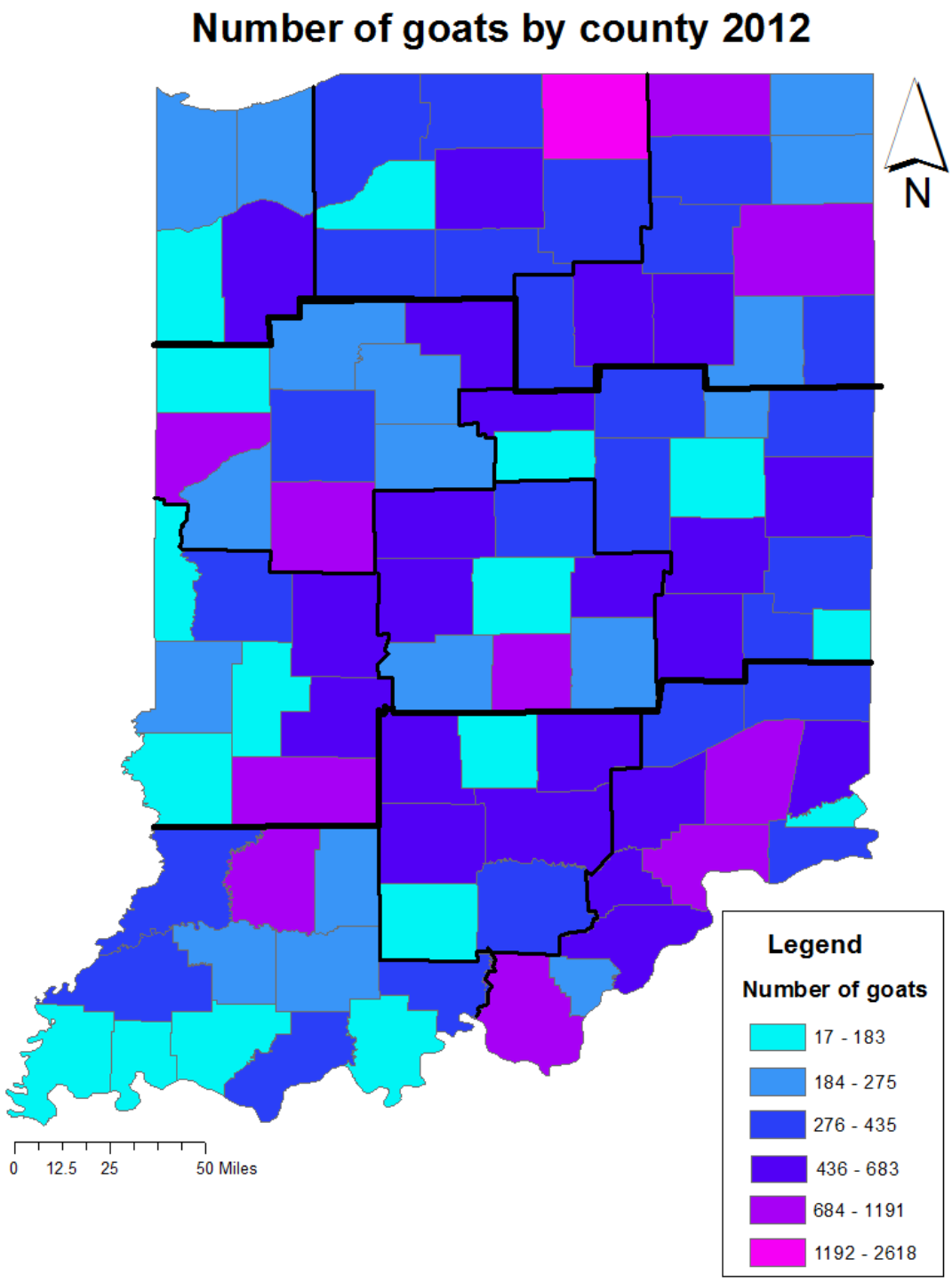

Figure 4-2. Geographic distribution of the Indiana goat population in 2012 based on the 2012 National Census of Agriculture (USDA, 2012).

Table 4-1. Number of goats by PHPD and region of Indiana in 2012 and the study sample

Region	PHPD	Total Goats	Percent of Indiana Goats	Goats in Sample	Percent of Sampled Goats
Northern	1	1502	3.9	32	4.9
	2	4595	11.9	32	4.9
	3	4818	12.5	141	21.7
Total		10915	28.3	205	31.5
Central	4	4054	10.5	75	11.6
	5	3734	9.7	89	13.7
	6	4444	11.5	86	13.2
	7	2953	7.6	31	4.8
Total		15185	39.3	281	43.3
Southern	8	2768	6.9	33	5.1
	9	6338	16.4	74	11.4
	10	3426	8.9	56	8.6
Total		12532	32.4	163	25.1
State Total		38632		649	

Table 4-2. Individual level prevalence estimates by PHPD and region

Region	PHPD	Positive	Negative	Total	Estimated Prevalence	95% CI
Northern	1	2	30	32	6.2%	1.7-20.1%
	2	4	28	32	12.5%	5.0-28.1%
	3	15	126	141	11.9%	7.3-18.7%
Total		21	184	205	10.2%	6.7-15.1%
Central	4	9	66	75	12.0%	6.4-21.3%
	5	8	81	89	9.0%	4.6-16.7%
	6	6	80	86	7.0%	3.2-14.4%
	7	0	31	31	0.0%	0-11.0%
Total		23	258	281	8.2%	5.5-12.0%
Southern	8	2	31	33	6.1%	1.7-19.6%
	9	16	58	74	20.3%	12.7-30.8%
	10	4	52	56	7.1%	2.8-17.0%
Region Total		22	141	163	13.5%	9.1-19.6%

Table 4-3. Herd level prevalence estimates by PHPD and region

Region	PHPD	Positive	Negative	Total	Estimated Prevalence	95% CI
Northern	1	2	4	6	33.3%	9.7-70%
	2	2	4	6	33.3%	9.7-70%
	3	4	15	19	21.0%	8.5-43.3%
Region Total		8	23	31	25.8%	13.7-43.2%
Central	4	4	8	12	33.3%	13.8-60.9%
	5	5	8	13	38.5%	17.7-64.5%
	6	2	8	10	20.0%	5.7-51%
	7	0	5	5	0.0%	0-43.4%
Region Total		11	29	40	27.5%	16.1-42.8%
Southern	8	2	4	6	33.3%	9.7-70.0%
	9	4	5	9	44.4%	18.9-73.3%
	10	2	6	8	25.0%	7.1-59.0%
Region Total		8	15	23	34.8%	18.8-55.1%

Table 4-4. Comparing the odds of an individual doe testing positive for *C. burnetii* in different PHPDs. PHPD 1 was used for comparison as the referent district and PHPD 7 did not have any does test positive for *C. burnetii*.

PHPD	OR	95%CI	p
1	NA	NA	NA
2	2.14	0.36-12.63	0.40
3	1.78	0.38-8.23	0.46
4	2.04	0.41-10.05	0.38
5	1.48	0.30-7.37	0.63
6	1.12	0.21-5.88	0.89
7	--	--	--
8	0.97	0.13-7.32	0.98
9	4.14	0.89-19.20	0.07
10	1.15	0.20-6.67	0.87

CHAPTER 5. EVALUATION OF THREE POTENTIAL RISK FACTORS FOR INFECTION WITH *COXIELLA BURNETII* IN GOATS IN INDIANA

5.1 Introduction

Coxiella burnetii is the causative organism of Q fever, a zoonotic disease that can lead to reproductive losses in ruminants and acute influenza-like disease, cardiovascular disease, chronic fatigue syndrome and pregnancy loss in people. The small cell variant of *C. burnetii* is environmentally resilient, allowing it to persist in a variety of environments (Kersh et al., 2010b), be carried as a fomite (Kersh et al., 2013) and be transmitted by wind (Tissot-Dupont et al., 2004). In addition, a wide variety of animal species have been documented to be infected by *C. burnetii* (Stein & Raoult, 1999; Cairns et al., 2007; Cooper et al., 2011; Kersh et al., 2010a; Reusken et al., 2011; Davoust et al., 2014).

C. burnetii can be considered to be a re-emerging zoonotic pathogen, defined as a disease or agent known to exist, but exhibiting an increase in incidence or range, (Morens et al., 2004). In some situations, *C. burnetii* also meets conditions that play a role in disease emergence such as environmental encroachment and an increased frequency of interactions between people and wildlife (Morens et al., 2004). An example of this is the recognition of the 3-toed sloth as a likely vector for a unique strain of *C. burnetii* in French Guiana (Davoust et al., 2014). However, the nonspecific clinical signs of infection with *C. burnetii* in both humans and animals make the

diagnosis of Q fever difficult and what could be viewed as re-emergence in some settings may simply be the result of underdiagnosis. These nonspecific clinical signs have contributed to the historically challenging nature of epidemiologic studies of *C. burnetii*.

Despite the wide range of host species and outbreaks of Q fever associated with other species (Pinsky et al., 1991; Stein & Raoult, 1999; Davoust et al., 2014), domestic ruminants are considered to be the main reservoir for human infection with *C. burnetii*. Small ruminants, in particular goats, have been associated with recent human outbreaks of Q fever in Europe and the United States (Schimmer et al., 2010; Bjork et al., 2014). Potential risk factors for *C. burnetii* infection in goats have been evaluated in the context of commercial farms (Schimmer et al., 2011). One of the inclusion criteria for that study was a herd size of at least 100 goats, but the median herd size was 782 goats. The seroprevalence of *C. burnetii* was 21.4% in the 2828 individual goats tested. Risk factors that were identified included: Other goat farms within 8km with *C. burnetii* in bulk tank milk, high density of cattle within the community, covering air spaces in the barns (either to control pests or as windbreaks), the presence of cats within the housing area and the use of artificial insemination. A herd size of 800 goats or more was also identified as a risk factor for *C. burnetii* infection at both the individual and herd levels (Schimmer et al., 2011).

While the seroprevalence of *C. burnetii* has been evaluated in goats at the state level in the United States (Sondgeroth et al.; 2013, Baker & Pithua, 2014), little work has been done investigating potential risk factors for *C. burnetii* infection in this species. Although there is regional variation, the 2012 Census of Agriculture reported 2,621,514

goats and 128,456 premises owning goats in the United States, resulting in an overall average of 20.4 goats per herd. In Indiana, 38,632 goats and 2883 premises were reported in 2012, resulting in an overall average of 13.4 goats per farm (USDA, 2012). Although the majority of the risk factors for *C. burnetii* identified by Schimmer et al are not related to herd size, there is the potential for variation in these risk factors depending on herd size and geographic location.

The purpose of this study was to investigate the role of 3 potential risk factors for *C. burnetii* infection in goats in Indiana. Infection with *C. burnetii* was defined in 2 ways: seropositivity for anti-*C. burnetii* antibodies through use of a commercially available enzyme linked immunosorbent assay (ELISA) and shedding of *C. burnetii* as detected by polymerase chain reaction (PCR) targeting the IS1111 transposon. These diagnostics were evaluated separately because seropositivity implies exposure to *C. burnetii* but not necessarily active infection in the absence of paired sera while shedding of *C. burnetii* DNA indicates active infection. The predictors of interest were: The presence of cattle, sheep or camelids on the farm, indoor housing and a history of reproductive events on the farm.

5.2 Materials and methods

5.2.1 Population of interest, recruitment and data collection

Individuals and herds in this study were identified as part of a cross-sectional, convenience sampled study to estimate the prevalence of *C. burnetii* in goats in Indiana. Goat does over 1 year of age and not pregnant at the time of sampling were chosen for inclusion in the study. Based on the reported 2883 premises with goats in Indiana (USDA,

2012) and utilizing an estimated herd level seroprevalence and DNA shedding prevalence of 50% and 95% confidence, a recruitment goal of 95 farms was identified. In order to determine the number of does to sample within a herd, an expected within herd seroprevalence of 40% and expected DNA shedding prevalence of 50% within an infected herd were utilized to calculate a sampling goal of 10 does per herd in herds with 100 does or fewer. In herds with more than 10 does meeting the inclusion criteria, the producer selected the individual does to be sampled. In herds with fewer than 10 does meeting the eligibility criteria, all eligible does were sampled.

Goat herds in Indiana were identified through personal communication at shows, the use of breed organization directories and through internet searches between May of 2012 and August of 2014. Producers were approached through face-to-face personal communication, e-mail and telephone conversations. The template for e-mail contacts can be found in Appendix A. Sample collection was then performed at shows or at scheduled farm visits.

Samples of blood, milk, vaginal mucus, and feces were collected on the same day from each doe selected from a given farm. The exception to this was a single farm that was recruited at a show. The does present at the show were sampled there and additional does were sampled at a subsequent farm visit. Sample collection techniques were approved by the Purdue Animal Care and Use Committee (protocol 1205000641) and were performed as follows: Blood samples were collected via jugular venipuncture by one of the investigators; milk samples were collected by the owner or an investigator utilizing the normal hygienic practices for milking at the farm; sterile cotton-tipped

plastic swabs were used by an investigator to collect samples of vaginal mucus; to ensure that the fecal samples represented a single doe, samples were collected from the rectum by an investigator unless the doe defecated at the time of sampling, in which case the immediately voided sample was collected. Samples were transported in coolers with ice or icepacks from the sampling location to Purdue University for laboratory analysis.

Two types of questionnaire, as approved by the Purdue University Institutional Review Board were presented to the owners of farms included in the study. These questionnaires are reproduced in Appendices B and C. The first (Appendix B) collected information about the farm and herd in general. The second (Appendix C) collected information about each specific doe included in the study. Topics covered in these questionnaires included:

1. Basic farm information including location (by county and zip code), length of ownership of the farm, length of ownership of goats, number and type of other animals present on the farm.

2. Demographic information on the goats owned including number of goats, breeds of goats, purpose of goats (milk production, meat production or fiber production), gender and ages of the goats and the number of goats introduced to the herd on a regular basis.

3. Husbandry information including outdoor versus indoor housing, indoor floor surface and use of veterinary care.

4. Reproductive disease history including abortions, stillbirths and weak kids for both the individual does and the farms as a whole.

5.2.2 Diagnostic methodology

Upon return to the laboratory at Purdue University, blood samples were centrifuged at 4000 rpm for 10 minutes to separate serum. After centrifugation, 1 to 2 ml of serum were removed from the collection tube and stored in cryotubes at -20^{o}C pending analysis. The Idexx Chekit Q-fever enzyme linked immunosorbent antibody test (ELISA) (trademark IDEXX Laboratories, Hoofddorp, the Netherlands) was utilized for evaluation of the presence of anti-*C. burnetii* antibodies. This ELISA detects antibodies to either phase I or phase II antigen (personal communication with customer service representative on the IDEXX Livestock and Poultry Team) with a reported sensitivity of 100% and specificity of 100%. When this test was compared with complement fixation in 81 samples, there was a correlation of 98% between the 2 types of tests (Schalch *et al.*, 1998). The sensitivity and specificity of the ELISA were independently estimated at the National Veterinary Services Laboratory using an expectation-maximization algorithm. The estimated sensitivity from this analysis was 97% and the estimated specificity was 100% (95% CI: 90-100%) (Emery et al., 2014). Samples were tested following the manufacturer's protocol using positive and negative controls provided by the manufacturer. A positive ELISA sample was defined by demonstration of an optical density (OD) at least 40% of that of the positive controls included with each assay. A negative sample was defined as one that demonstrated an OD less than 30% of that of the positive control after correction by subtraction of the OD of the negative control. Samples with OD between 30 and 40% of the positive control after correction by

subtraction of the negative control were defined as indeterminate and retested. None of the samples remained indeterminate after subsequent testing.

Upon return to the laboratory at Purdue University, milk, vaginal swab and fecal samples were stored at -20^{o}C pending DNA extraction. If DNA extraction was performed within 24 hours of collection milk samples were stored under refrigeration. DNA was extracted from the milk samples utilizing a Qiagen DNeasy Blood and Tissue Kit (trademark Qiagen, Valencia, California) as previously described (Bauer et al., 2015). DNA samples were stored at -20^{o}C prior to real time quantitative polymerase chain reaction (PCR) testing.

A Qiagen DNeasy Blood and Tissue Kit (trademark Qiagen, Valencia, California) protocol for buccal DNA extraction was utilized for DNA extraction from the vaginal swabs. Swabs were suspended in 400µl sterile PBS. After suspension, 20µl Proteinase K and 400µl Buffer AL were then added to each sample. Swabs and reagents were mixed by vortex, incubated at 56^{o}C for 10 minutes and briefly centrifuged prior to adding 400µl ethanol to the lysate. The lysate was filtered through columns and washed with buffers AW1 and AW2. Buffer AE (150µl) was added to the columns for the final elution. DNA samples were stored at -20^{o}C prior to PCR testing.

A QIAamp DNA Stool Mini Kit (trademark Qiagen, Valencia, California) was utilized for DNA extraction from fecal samples. Individual sample aliquots of 200mg of feces were used for each extraction. One ml of Inhibitex Buffer was added to each sample, vortexed to mix and incubated at 95^{o}C for 5 minutes prior to centrifugation.

After centrifugation, 200µl of supernatant was added to 15µl Proteinase K and 200µl of Buffer AL was added. Samples were vortexed and incubated at 70^{o}C for 10 minutes. After incubation, 200µl ethanol was added to the lysate, mixed and 600µl of the lysate was filtered through columns and washed with buffers AW1 and AW2. Buffer ATE (200µl) was used for the final elution. DNA samples were stored at -20^{o}C prior to PCR testing.

Real time quantitative PCR was used to identify the presence of an 86 base pair (bp) region of the *C. burnetii* IS1111 transposon (Panning et al., 2008) as previously described (Bauer et al., 2015). A Stratagene Mx3000P qPCR system (trademark Agilent Technologies, Santa Clara, CA) was utilized for real-time PCR and results were collected in MxPro (trademark Agilent Technologies, Santa Clara, CA). PCR was repeated for samples demonstrating a cycle threshold (CT) value and samples with an average CT value less than or equal to 36.5 were defined as positive for analytic purposes. Individuals testing positive for *C. burnetii* DNA from any of the 3 samples were defined as positive individuals for analytic purposes.

5.2.3 Statistical analysis

Two types of cases were defined. Seropositive cases had an OD greater than or equal to 40% of that of the controls as described above, indicating that an immune response against *C. burnetii* was mounted by the doe at some point in her life. DNA positive cases had an average CT values less than or equal to 36.5 from any of the samples tested by PCR, indicating active shedding of *C. burnetii* at the time of sampling.

Logistic regression models were constructed in SPSS (SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp Released 2013) to evaluate the effects of 3 predictors of interest on these outcomes. The predictors of interest were: Housing in confinement, the presence of other ruminants (cattle, sheep or camelids) on the farm and the past occurrence of reproductive events in the goats on the farm. For the purposes of the current study, confinement was defined as being housed indoors with very limited to no outdoor access. Since *C. burnetii* is primarily transmitted by inhalation, being housed primarily indoors could increase the chances of a doe encountering the organism in dust within the barn as compared to living in an outdoor environment with a greater degree of air circulation. Cattle and sheep are known reservoir species for *C. burnetii* (Rodolakis et al, 2007). Llamas and alpacas are kept as livestock guardian animals. The presence of other reservoirs on the farm and potentially in close proximity to goats would be expected to facilitate transmission of *C. burnetii*. Does living on farms where reproductive events have occurred could be expected to have a greater risk of exposure to *C. burnetii* in the environment than does living on farms where reproductive events have not occurred. For the purposes of this study, a reproductive event was defined as abortion, stillbirth or weak kids at the time of parturition.

Covariates to be included in model construction were defined for each of the predictors of interest through the use of causal diagrams (Dohoo et al., 2009). Figure 1 illustrates the causal diagram initially constructed to identify the covariates of interest for each predictor of interest. Backward selection based on a 10% change in the odds ratio for the predictor of interest was utilized to select influential confounders.

5.3 Results

5.3.1 Study sample and variables of interest

A total of 654 does representing 95 farms were included in the study. Serologic results were available for 613 does. At least one type of sample was tested for *C. burnetii* DNA in all 654 does (Table 5.1). Milk, vaginal discharge and fecal samples were all tested in 349 does. Of the does where all 3 types of samples were not collected, 31 does provided both vaginal and milk samples but not fecal samples, 226 does provided both vaginal and fecal samples but not milk samples and 1 doe provided both milk and fecal samples but a vaginal sample was not collected. Forty-five does had only a single sample type tested for *C. burnetii* DNA. Serum samples from 23 does (3.8%) tested positive for anti-*C. burnetii* antibodies. A total of 49 does (7.5%) tested positive for *C. burnetii* DNA by at least 1 route of shedding.

There was information available for 2 of the 3 predictors of interest for all 654 of the does sampled (Table 5.2). Two-hundred-forty-six does (40.4%) lived on farms where other ruminants were housed. Of these 246, 118 (48.0%) lived on farms where cattle were the only other ruminant species, 59 (20%) lived on farms where sheep were the only other ruminant species and 41 (16.7%) lived on farms where camelids were the only other ruminant species. Cattle and sheep were both present on the farms where 25 does lived (10.2%), cattle and camelids were present on the farms where 3 does lived (1.2%). No does lived on farms where both sheep and camelids were present. Of 654 does sampled, 626 (95.7%) lived on farms where reproductive events had occurred. Housing information was available for 644 of the 654 does sampled. Seventy-one does

(11.0%) were housed primarily indoors and 573 does (89.0%) had outdoor access at least 50% of the time. Of these 583 does, 211 (36.8%) were housed primarily outdoors.

Potential confounding factors were identified for all of the predictors of interest in this study. For indoor housing, breed type and the presence of other ruminants on the farms were identified as potential confounders. For cattle, sheep or camelids on the farm, breed type and herd size were identified as potential confounders. For herd level reproductive events, the presence of cattle, sheep or camelids was identified as a potential confounding factor.

5.3.2 Model building

Information on housing was available for 606 of the 613 does tested by ELISA (Table 5.2). Seventy of these does were housed primarily indoors (11.5%). Only 1 of the does tested positive for anti-*C. burnetii* antibodies by ELISA. All 613 of the does tested by ELISA had information about the presence of cattle, sheep or camelids on the farm available. Cattle, sheep or camelids were present on the farms where 231 does lived (37.7%). Three of these does tested positive for anti-*C. burnetii* antibodies by ELISA. Information on the history of reproductive events on the farm was available for all 613 of the does tested by ELISA. Five-hundred-eighty seven does (95.6%) lived on a farm where reproductive events had occurred. All of the does that tested positive for anti-*C. burnetii* antibodies resided on farms with a history of reproductive events. A value of 1 was added to each of the cells to attempt odds ratio estimation. Table 5.2 also includes the univariate analyses of the predictors of interest. The presence of cattle, sheep or camelids on the farms was the only predictor of interest that was statistically

significantly related to seropositivity for *C. burnetii*. Does housed on farms with these other species present demonstrated a 5-fold decrease in the odds of testing positive for anti-*C. burnetii* antibodies.

Three separate logistic regression models were constructed to evaluate the relationship between each of the predictors of interest and seropositivity for *C. burnetii*. Table 5.3 provides information on the covariates included in the final models after backward selection was implemented. In these models, only the presence of other ruminants on the farm had a statistically significant association with the detection of anti-*C. burnetii* antibodies in individual does (OR = 0.23, 95% CI: 0.07 – 0.79, p = 0.020).

Information on housing was available for 644 does tested for C. burnetii by PCR (Table 5.2). Seventy-one of these does (11.0%) were housed primarily indoors, including 6 of the 49 does positive for shedding *C. burnetii* DNA (12.2%). Information on the presence of cattle, sheep or camelids on the farm was available for all 654 does. Two-hundred and forty-six of these does (37.6%) lived on farms that housed other species of domestic ruminants and 7 of the 49 positive does (14.3%) were among this group. Information about the history of reproductive events on the farm was available for 654 does. Six-hundred-twenty-six of the does (95.7%) lived on farms where a reproductive event had occurred. Forty-seven of the does positive for shedding *C. burnetii* DNA (95.9%) lived on a farm where a reproductive event had occurred. Table 5.2 details the univariate analyses of the predictors of interest. The presence of cattle, sheep or camelids on the farms was the only predictor of interest that was statistically significantly related to shedding of *C. burnetii*, with does living on farms with these

other species present demonstrating a 70% decrease in the odds of testing positive for anti-C*. burnetii* antibodies.

Three separate logistic regression models were constructed to evaluate the relationship between each of the predictors of interest and shedding of *C. burnetii* by individual does. Table 5.4 provides information on the covariates included in the final models after backward selection was implemented. In modelling the presence of cattle, sheep or camelids on the farm, neither of the proposed confounders was influential and thus the crude odds ratios are reported. In these models, only the presence of other ruminants on the farm had a statistically significant association with shedding of *C. burnetii* with a 3.8-fold decrease in the odds of *C. burnetii* DNA being detected in does living on farms with cattle, sheep or camelids present as compared to does living on farms without these species present (OR = 0.26, 95% CI: 0.11 – 0.58, p = 0.001).

5.4 Discussion

This study utilized both molecular and serologic data to evaluate factors potentially associated with *C. burnetii* infection. The use of both molecular and serologic diagnostics to evaluate potential risk factors for *C. burnetii* infection is a unique aspect of this study. The development of culture techniques (Omsland et al., 2009) has improved the understanding of the genetics of *C. burnetii*, leading to molecular diagnostic methods that are both rapid and sensitive. In addition, understanding the biphasic nature of the immune response to *C. burnetii* has allowed the development of sensitive methods for detection of exposure to the bacteria through serology (Emery et al., 2012).

Due to the lack of seropositive does on farms where reproductive events had not occurred, history of reproductive events within the herd was only included as a predictor in the model of DNA shedding. This does not mean that a history of reproductive events on the farm is negligible in relationship to exposure to *C. burnetii*. Rather, this lack of seropositive does on farms without a history of reproductive events warrant further investigation. However, a sample with a greater prevalence of anti-*C. burnetii* antibodies is needed and a case-control design would be more appropriate. Further investigation into the relationship between prior reproductive events and exposure to *C. burnetii* would also benefit from defining the history of reproductive events in a time-restricted manner (i.e. within the past 2 years) as events that occurred more recently would be expected to have a greater likelihood of infecting previously unexposed does. However, several other pathogens can result in late term abortion in goats, including *Toxoplasma gondii*, *Chlamydophila abortus*, *Listeria monocytogenes*, *Brucella melitensis* and *Leptospira interrogans* (serovars Grippotyphosa and Pomona) (Merck, 2005). Coinfection with *C. burnetii*, *T. gondii*, and *C. abortus* has been documented in cases of abortion in goats (Hazlett et al., 2013). A history of reproductive events may be tied to another pathogen even if *C. burnetii* is identified in a doe or herd and it is possible that even in an ideal sample the history of reproductive events on a farm may not be associated with increased odds of detecting *C. burnetii*.

Indoor housing did not prove to be a statistically significant predictor in relationship to either seropositivity or DNA shedding. The small sample size, in particular the low prevalence of *C. burnetii* in this sample, likely precluded conclusions being

drawn as to the effect of this variable. Indoor housing was chosen as a risk factor for exploration because inhalation is the main route of *C. burnetii* transmission and goats housed in indoor spaces may be exposed to higher concentrations of *C. burnetii* in dust that goats that are outdoors. The use of wind curtains or wind breaks was previously identified as a risk factor for seropositivity in goats (Schimmer et al., 2011) and *C. burnetii* DNA has been identified in goat pens up to 1 year after an outbreak (Kersh et al., 2013). In this study, the goats were mainly kept in small herds (median herd size 20 goats) and the largest herd (150 goats) had equal access to indoor and outdoor environments. Further studies on the role of indoor housing may benefit from focusing on commercial operations which are more likely to house goats in a truly indoor facility, have larger herd sizes, and utilize wind screens or similar measures for protection from adverse weather or wildlife.

The presence of cattle, sheep or camelids on the farm was associated with a statistically significant decrease in the odds of both testing positive for anti-*C. burnetii* antibodies (5-fold decrease) and shedding of *C. burnetii* DNA (4-fold decrease) in this study. The direction of this association is surprising, but may be due to a tendency for farms with a large variety of animal species to have fewer numbers of each species. However, the size of the goat herd on farms with other ruminants ranged from 1 to 150 goats which is not consistent with the explanation of a smaller goat herd size on farms with cattle, sheep or camelids in addition to goats. Alternately, species dominant strains of *C. burnetii* may not transmit as efficiently between ruminant species. There has been documentation of the proposed cattle dominant sequence type of *C. burnetii* (ST20) in

goat milk (Pearson et al., 2014) and the proposed goat dominant sequence type (ST8) in cattle bulk tank milk samples (Bauer et al., 2015). An ecological phenomenon called the dilution effect provides another potential explanation. A dilution effect occurs when the presence of a wide variety of potential host species with a poor ability to transmit the pathogen of interest in addition to more competent host species decreases the risk of pathogen transmission (Schmidt & Ostfeld, 2001). For density-dependent pathogens, adding alternate host species can decrease the risk of disease transmission if between species transmission occurs less frequently than within species transmission (Keesing et al., 2006). However, more work is needed to elucidate transmission patterns for *C. burnetii* between species in a community such as a farm.

Although not defined as a predictor of interest in this study, one covariate was influential in relationship to the predictors of interest and may be worth evaluating as a risk factor in future studies. Whether or not a doe was from a meat breed as compared to a non-meat breed was included as a covariate in 4 of the 6 models as a surrogate for unmeasured intrinsic factors (e.g. immune function, differences in mammary structure at the microscopic level) and unmeasured extrinsic factors (e.g. density, diet variation) when compared with other types of breeds. Meat breed was retained as a covariate in the models in which indoor housing was the predictor of interest and in the model evaluating the relationship between seropositivity and the presence of cattle, sheep or camelids on the farm. Although not within the scope of this study, investigating breed and breed class variation in factors such as leukocyte numbers, macrophage activity and

T-cell activity could help to determine if there are intrinsic differences in the ability of different types of goats to respond to infection with *C. burnetii*.

Limitations of this study include small sample size, lack of information on the density in which the goats were housed on each farm and self-selection bias through use of a convenience sample. Low prevalence of *C. burnetii* infection in the sample limits the power of this study to detect weak associations. Because of this, the results of this analysis should be considered as guides for the development of future avenues of research. Density may be more important than its surrogate factors type of housing and number of goats in evaluating risk factors for *C. burnetii* infection. Infected goats kept at a higher density would result in a greater amount of bacterial contamination per square meter than infected goats kept at lower densities. The area of the space on each farm set aside for goat housing was not measured in this study and thus density could not be calculated. Finally, producers determined whether or not to participate in this study and also selected which does would be tested in situations where more than 10 eligible does were present in a herd. The selection bias introduced by this process could have resulted in overestimation of prevalence if producers with does exhibiting reproductive events were more likely to volunteer for inclusion in the study than producers owning herds where reproductive events had not occurred. At the individual level, producers may have been more likely to select does with a history of reproductive events for inclusion. This would also result in an overestimation of prevalence and the associations detected in this study may not be as strong as reported. Random selection of does to be sampled would have helped to control this bias. *C. burnetii* is a reportable disease in

Indiana. Although farm information was recorded by number rather than name to preserve anonymity, test results were reported to the ISBAH at the zip code and county level. Reluctance to have results reported may have influenced some producers to decline participation in this study, resulting in an underestimation of the prevalence of *C. burnetii*. Underestimation of prevalence may have led to underestimation of the strength of the associations reported in this study. In future studies of this nature, it may be helpful to include a question on reasons for declining participation in order to better clarify the direction of this bias.

In this study, does living on farms on which cattle, sheep or camelids were present was associated with decreased odds of testing positive for past exposure to and current shedding of *C. burnetii*. This finding was unexpected, but may be due to a dilution effect. Whether the protective association found in this study could be attributable to a dilution effect deserves further investigation. The factors influencing *C. burnetii* transmission are complex and a full picture of pathogen epidemiology and ecology must incorporate multiple reservoir and host species within the community of interest, intrinsic host factors such as immunocompetence and abiotic factors such as wind.

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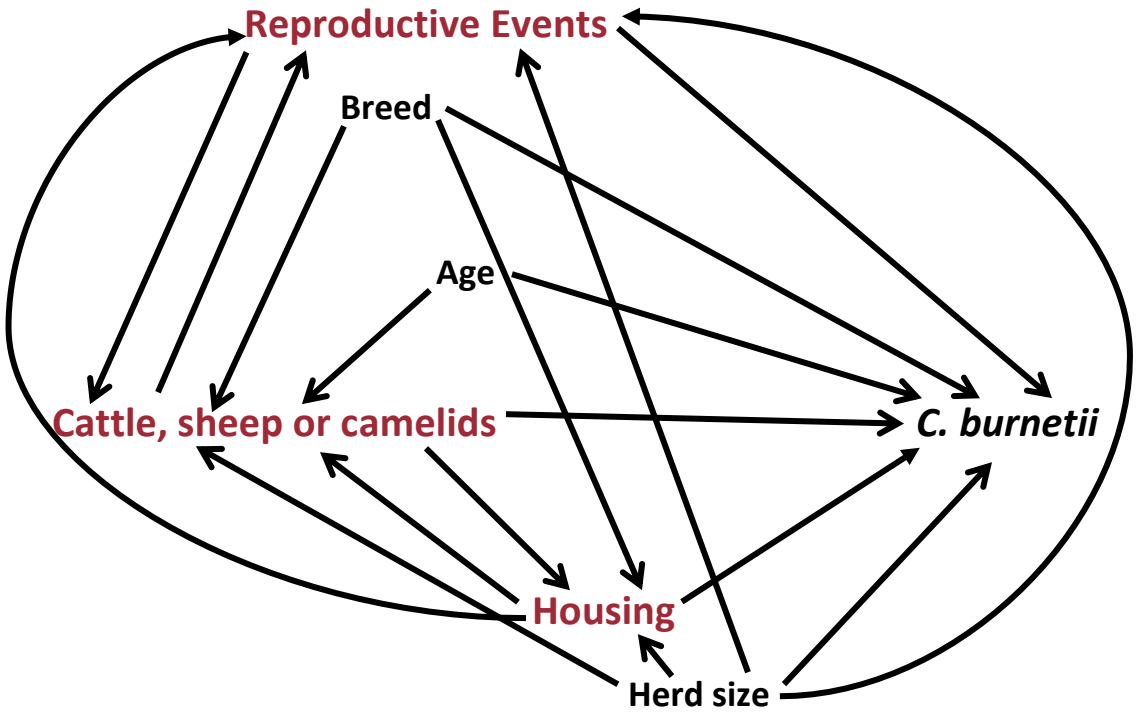

Figure 5-1. Causal diagram illustrating the relationships between the predictors of interest (red), covariates of interest and infection with *C. burnetii*.

Table 5-1. Diagnostic tests and results

Testing Method	ELISA		PCR	
	Serum	Milk	Vaginal Mucus	Fecal Material
Number Positive	23	9	40	1
Number Negative	590	382	597	578
Total Tested	613	391	637	579

Table 5-2. Univariate analysis of the predictors of interest. * indicates that 1 was added to each cell during the analysis in order to estimate the odds ratio

Diagnostic Method			Positive	Negative	Total	Univariate Odds Ratio	95% CI	p-value
	Indoor housing	Yes	1	69	70	0.34	0.04-2.55	0.30
		No	22	514	536			
		Total	23	583	606			
ELISA	Cattle, sheep or camelids on the farm	Yes	3	228	231	0.24	0.07-0.81	0.022
		No	20	362	382			
		Total	23	590	613			
	Reproductive event on the farm	Yes	23	564	587	1.15*	0.15-8.81*	0.99
		No	0	26	26			
		Total	23	590	613			
	Indoor housing	Yes	6	65	71	1.14	0.47-2.78	0.74
		No	43	530	573			
		Total	49	595	644			
PCR	Cattle, sheep or camelids on the farm	Yes	7	239	246	0.26	0.12-0.58	0.0002
		No	42	366	408			
			49	605	654			
	Reproductive event on the farm	Yes	47	579	626	1.06	0.24-4.58	0.94
		No	2	26	28			
		Total	49	605	654			

Table 5-3. Results for models with seropositivity as the outcome of interest

Predictor	Covariates in model	Odds ratio	95% Confidence Interval	p-value
Indoor housing		0.31	0.04-2.33	0.25
	Meat breed	0.62	0.24-1.60	0.32
Cattle, sheep or camelids on the farm		0.23	0.07-0.79	0.020
	Meat breed	0.61	0.23-1.57	0.30

Table 5-4. Results for models with shedding of *C. burnetii* DNA as the outcome of interest

Predictor	Covariates in model	Odds ratio	95% Confidence Interval	p-value
Indoor housing		0.93	0.39-2.36	0.93
	Meat breed	0.36	0.17-0.80	0.012
Cattle, sheep or camelids on the farm		0.26	0.11-0.58	0.001
Reproductive event on the farm		0.82	0.18-3.67	0.80
	Cattle, sheep or camelids on the farm	0.25	0.11-0.57	0.001

CHAPTER 6. CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Conclusions

The goal of this project was to establish baseline information about *C. burnetii* infection in goats in Indiana. Given that previous studies using serologic techniques (Sondgeroth et al., 2013; Baker & Pithua, 2014) and outbreak investigations (Bjork et al., 2014) have established goats as reservoirs for *C. burnetii* in the United States, the presence of *C. burnetii* in goats in Indiana is not unexpected. At the individual level, 3.8% of does in this sample showed serologic evidence of exposure to *C. burnetii*. DNA shedding by at least 1 route was detected in 7.5% of the does in this study. At the farm level, 11% of the recruited farms had at least one doe with antibodies to *C. burnetii*. Twenty percent of the study farms had at least 1 doe test positive for shedding of *C. burnetii* DNA. Although these numbers may not seem overwhelming and sample size limits generalization, based on these numbers 577 of the 2883 farms registered in 2012 may have housed goats shedding *C. burnetii*. With an infectious aerosol dose of between 1 and 10 organisms (Oyston & Davies, 2011), *C. burnetii* in goats presents a potential public health risk in Indiana.

Although sample size is a limiting factor in generalizing findings about the geographic distribution of caprine *C. burnetii* in Indiana, the greater proportion of

positive animals in PHPD 9 provides a geographic target for future research into *C. burnetii*. The use of Cohen's kappa as a measure of association between serologic and molecular diagnostic tests was compromised by the low overall prevalence of *C. burnetii* infection in Indiana. Given the results of previous experimental and field studies (Arricau-Bouvery *et al.*, 2003; Rousset *et al.*, 2009), this lack of association is likely a true finding. Focusing future studies in a geographic area where there is higher prevalence of infection or utilizing a case-control study design would provide additional support for this conclusion.

In this study, goats living on farms with cattle, sheep or camelids had a reduction in the odds of testing positive for *C. burnetii*. This may be due to the dilution effect. In a study evaluating the dilution effect in the context of Lyme disease, increased biodiversity in the environment was found to have a protective effect against human infection with *Borrelia burgdorferi* (LoGiudice et al., 2003). Although ticks are considered to be vectors for *C. burnetii* and the dilution effect was first explored in the context of vector borne disease, transmission within domestic ruminant herds and between ruminants and people is most commonly through inhalation of aerosolized bacteria rather than by vectors. *C. burnetii* may act as a density-dependent pathogen where additional host species decrease the risk of transmission if between species transmission occurs less frequently than within species transmission, but in order to clarify this relationship studies involving all potential host species on a given farm are needed.

While it is disappointing that genotyping was not effective for a greater number of samples in this study, identifying ST8 in 3 samples is consistent with the hypothesis that this sequence type is dominant in goats in the United States (Pearson et al., 2014). Incorporating culture into the genotyping process may be of use in future studies. Alternatively, pooled milk samples or pelleting milk solids for use in extraction may help to increase the concentration of *C. burnetii* DNA and the likelihood of successful sequence typing.

6.2 Future directions

Although the focus of these studies was on *C. burnetii* as an infectious disease in goats, it cannot be removed from the context of public health. Because of this, there are 2 future directions for this work: research and outreach. Research through a combination of field and laboratory studies is important because there is still much to learn about the epidemiology of *C. burnetii*. Outreach is important because with the lack of approved vaccinations for animals and the general public in the United States, prevention and control of *C. burnetii* infection is dependent upon the knowledge of veterinarians and physicians and their ability to convey that knowledge to individuals at risk for exposure to *C. burnetii*.

In the context of future research, there are also 2 important directions. The first of these involves investigation of the molecular epidemiology of *C. burnetii*. Protocols for rapid sequence typing are available (Hornstra et al., 2011). The use of these could greatly expand the understanding of *C. burnetii* in the context of clinical disease, if sequence typing was attempted on every diagnostic sample testing positive for *C.*

burnetii. Both ST20 and ST8 have been identified in bulk tank milk samples from cattle in Indiana (Bauer et al., 2015), but the small number of samples from goats successfully sequence typed in this study contributes little to the molecular epidemiology of *C. burnetii* in Indiana and no work investigating the prevalence or genotypes of *C. burnetii* in sheep in Indiana has been performed. A second route for future research involves investigating the role of community structure in transmission of *C. burnetii*. In the current project, the presence of cattle, sheep and camelids on goat farms resulted in a decreased odds of does testing positive for both exposure to *C. burnetii* and shedding of *C. burnetii* DNA. Although experimental work evaluating the routes of shedding of *C. burnetii* in cattle, goats and sheep has been performed (Rodolakis et al., 2007), no field studies have been conducted to evaluate infection with *C. burnetii* in multiple species on the same farm. Most publications on the role of host species beyond sheep, cattle and goats in transmission of *C. burnetii* have been case reports (Pinsky et al., 1991; Stein & Raoult, 1999). A prospective study including all domestic mammalian species on multiple farms, the people living and working with them and any external parasites identified would be very helpful in gaining better understanding of the transmission cycles of *C. burnetii*.

Outreach efforts are important to improve detection and treatment of Q fever. Although 20% of the goat farms in this study had at least 1 doe shedding *C. burnetii,* Q fever appears to be an uncommon diagnosis in Indiana (McQuiston et al., 2006; Beall et al., 2007). Nevertheless, increasing veterinarian and physician awareness of *C. burnetii* in Indiana could serve to improve diagnosis and reporting of Q fever. This would

enhance the effectiveness of passive surveillance system currently used for Q fever reporting in Indiana. In addition to focusing outreach and educational efforts on medical professionals, increasing producer awareness of Q fever would be beneficial. Educating producers about *C. burnetii* would allow them to protect their own health through the use of personal protective equipment. Awareness of Q fever may also encourage producers to seek medical treatment if they develop an influenza-like illness. PHPD 9 may be a good place in Indiana to develop and pilot test programs aimed at educating producers about the risks of infection with *C. burnetii* and the actions that they can take to protect themselves.

Q fever is a disease that the people and animals of Indiana will be living with for a long time. The studies comprising this project have laid a foundation for understanding *C. burnetii* in Indiana, but it is certainly not a completed process. Other species, including but not limited to sheep, camelids and people needed to be included in the structure that may eventually encompass the complete epidemiology of *C. burnetii* in Indiana.

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APPENDICES

Appendix A: E-mail recruitment letter

Dear Goat Owner,

I am a graduate student at Purdue studying a disease called Q fever and its presence in Indiana. Q fever is a disease of ruminants (goats, sheep and cattle) and humans. In ruminants the primary sign of Q fever is abortion. In people it can cause a flu-like illness, pregnancy loss in women and in some cases long term heart problems or chronic fatigue syndrome. *Coxiella burnetii*, the Q fever bacterium, is shed in reproductive discharges, milk, feces and urine. Humans are exposed to Q fever through inhaling or ingesting bacteria from these sources.

I would like to determine the prevalence of Q fever in goats in Indiana. In order to do this, I hope to collect samples of blood to test for previous exposure to Q fever and milk, vaginal swabs and feces to check for active shedding of bacteria. I will be sampling from up to 10 does over 1 year of age per farm, excluding pregnant does. In addition, I would like to evaluate risk factors for Q fever. Evaluating risk factors involves completing one survey on farm and management practices and a second survey for data about each animal that we sample such as age, breed, reproductive history, etc.

Your information will remain confidential. At the time of enrollment in the study, you will receive a farm identification number and each doe will receive an animal identification number. Any information that we publish will be based on the county, region (northern, central or southern) and the state of Indiana. Individual farm names and locations will not be disclosed. You may choose to learn the test results of your animals, but you are not obliged to. If you choose to learn the test results, you can

contact me via telephone or e-mail and provide me with your farm identification number. Test results will only be identified by this number. You can choose to withdraw from the study at any time without penalty.

Our goal is to gain basic information on the prevalence and distribution of Q fever in Indiana while protecting producer confidentiality. If you would be interested in participating in this study or have additional questions, please contact me at bauer20@purdue.edu.

Sincerely,

Dr. Amy Bauer

Graduate Student

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College of Veterinary Medicine

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(765)494-2294

Appendix B: Animal history survey

Animal Sample Form (Fill out 1 form for each animal sampled)

1. Date _____/_____/2014
2. Farm ID (match Farm Survey) _____
3. Animal ID (Farm ID –Animal ID) _____
4. Breed (circle one – should be just for the animal being sampled)
    a. Alpine
    b. LaMancha
    c. Nubian
    d. Toggenburg
    e. Saanen
    f. Oberhasli
    g. Sable
    h. Nigerian Dwarf
    i. Other _____
5. Age _____ Years
6. Most recent kidding? (Month) _____
7. How many times has she kidded?_____
8. Any history of abortion with this goat?

    Yes No

    a. If yes, what year(s)? _____
    b. Was a diagnosis made?

        Yes No

    c. What was the diagnosis?_____

9. Any history of stillbirth with this goat?

    Yes No

    a. If yes, what year(s)?

b. Was a diagnosis made?

Yes No

c. What was the diagnosis?_____

10. Any history of weak kids with this goat?

Yes No

a. If yes, what year(s)? _____

b. Was a diagnosis made?

Yes No

c. What was the diagnosis?_____

11. Any history of resorption with this goat?

Yes No

a. If yes, what year(s)? _____

b. Was a diagnosis made?

Yes No

c. What was the diagnosis?_____

Appendix C: Farm information survey:

Indiana Goat Owner Survey

1. Date _____/_____/2014
2. Farm ID _____
3. Farm Zip code _____
4. Farm County _____
5. No. Animals Sampled _____
6. Location of Interview
    a. Farm_____
    b. Fair _____
    c. Other _____
7. How long have you owned your farm? _____ years
8. For how many years have you owned goats? _____
9. How many goats do you own? _____
    a. Number of bucks: _____
    b. Number of wethers: _____
    c. Number of does: _____
10. How many different breeds do you have? _____
11. What breeds are they (dairy, meat or other – circle all that apply)?
    a. Alpine
    b. LaMancha
    c. Nubian
    d. Toggenburg
    e. Saanen
    f. Oberhasli
    g. Sable
    h. Nigerian Dwarf
    i. Other Dairy Breed _____

j. Boer Meat

k. Kiko Meat

l. Tennessee/Fainting/Myotonic

m. Spanish Meat

n. Other Meat Breed _____

12. What do you use the goats for?

a. Meat Yes No

b. Pasteurized Milk Yes No

c. Pasteurized Cheese Yes No

i. What type(s) of cheese _____

d. Raw Milk Yes No

i. For animal consumption Yes No

ii. For human consumption Yes No

e. Raw Cheese Yes No

i. What type(s) of cheese _____

f. Soap Yes No

g. Companions/Pets Yes No

h. Showing or 4-H Yes No

i. Other _____

13. Do you own other animals? Yes No

a. Sheep Yes No

b. Cattle Yes No

i. If yes, are these dairy cattle? Yes No

c. Alpaca/llamas Yes No

d. Cats Yes No

e. Dogs Yes No

f. Horses Yes No

g. Chickens Yes No

h. Other _____

14. Are you the person who primarily takes care of the goats? Yes No

15. Do you have non-family employees that work with the goats? Yes No

    a. How many? _____

    b. Do they have goats of their own? Yes No Don't know

16. Have you ever experienced abortions in your goats? Yes No

    a. If yes, in which year were the most recent abortions? _____

    b. How many aborted at that time? _____

    c. Was a diagnosis made? Yes No

    d. What was the diagnosis?

    _____

    e. How do you dispose of the abortive material [how is it handled (gloves, hands, shovel) and where does it go]?

    _____

    _____

    _____

    _____

17. Have you ever experienced abortions in your sheep? Yes No

    a. If yes, in which year were the most recent abortions? _____

    b. How many aborted at that time? _____

    c. Was a diagnosis made? Yes No

    d. What was the diagnosis?

    _____

    e. How do you dispose of the abortive material [how is it handled (gloves, hands, shovel) and where does it go]?

    _____

    _____

    _____

    _____

18. Have you ever experienced abortions in your cattle? Yes No

    a. If yes, in which year were the most recent abortions? _____

    b. How many aborted at that time? _____

    c. Was a diagnosis made? Yes No

    d. What was the diagnosis?

    _____

    e. How do you dispose of the abortive material [how is it handled (gloves, hands, shovel) and where does it go]?

    _____

    _____

    _____

    _____

19. Have you ever experienced stillbirths in your goats? Yes No

    a. If yes, in which year were the most recent stillbirths? _____

    b. How many stillbirths occurred at that time? _____

    c. Was a diagnosis made? Yes No

    d. What was the diagnosis?

    _____

    e. How do you dispose of the stillborn material [how is it handled (gloves, hands, shovel) and where does it go]?

    _____

    _____

    _____

    _____

20. Have you ever experienced stillbirths in your sheep? Yes No

    a. If yes, in which year were the most recent stillbirths? _____

    b. How many stillbirths occurred at that time? _____

    c. Was a diagnosis made? Yes No

d. What was the diagnosis?

_____

e. How do you dispose of the stillborn material [how is it handled (gloves, hands, shovel) and where does it go]?

_____

_____

_____

_____

21. Have you ever experienced stillbirths in your cattle? Yes No

a. If yes, in which year were the most recent stillbirths? _____

b. How many stillbirths occurred at that time? _____

c. Was a diagnosis made? Yes No

d. What was the diagnosis?

_____

e. How do you dispose of the stillborn material [how is it handled (gloves, hands, shovel) and where does it go]?

_____

_____

_____

_____

22. Have you ever had weak kids from your goats? Yes No

a. If yes, in which year were the most recent weak kids born?

_____

b. How many weak kids were born at that time?

_____

c. How many weak kids survived?

_____

d. Was a diagnosis made? Yes No

e. What was the diagnosis?

_____

f. How were any kids that died disposed of?

_____

_____

23. Have you ever had weak lambs from your sheep? Yes No

a. If yes, in which year were the most recent weak lambs born?

_____

b. How many weak lambs were born at that time?

_____

c. How many weak lambs survived?

_____

d. Was a diagnosis made? Yes No

e. What was the diagnosis?

_____

How were any lambs that died disposed of?

_____

_____

_____

24. Have you ever had weak calves from your cattle? Yes No

a. If yes, in which year were the most recent weak calves born?

_____

b. How many weak calves were born at that time?

_____

c. How many weak calves survived?

_____

d. Was a diagnosis made? Yes No

e. What was the diagnosis?

_____

f. How were any calves that died disposed of?

_____

_____

_____

25. Have you ever had any pregnancies resorbed in your goats? Yes No

a. If yes, in which year were the most recent resorptions?

_____

b. How many resorptions occurred at that time?

_____

c. Was a diagnosis made? Yes No

d. If yes, what was the diagnosis?

_____

e. Have you been able to breed the doe(s) successfully since this time? Yes No

26. Have you ever had any pregnancies resorbed in your sheep? Yes No

a. If yes, in which year were the most recent resorptions?

_____

b. How many resorptions occurred at that time?

_____

c. Was a diagnosis made? Yes No

d. If yes, what was the diagnosis?

_____

e. Have you been able to breed the ewe(s) successfully since this time? Yes No

27. Have you ever had any pregnancies resorbed in your cattle? Yes No

a. If yes, in which year were the most recent resorptions?

_____

b. How many resorptions occurred at that time?

_____

c. Was a diagnosis made? Yes No

d. If yes, what was the diagnosis?

_____

e. Have you been able to breed the cow(s) successfully since this time? Yes No

28. How do you breed your goats?

a. Natural – goats on premises Yes No

b. Natural – non-owned goats Yes No

i. Does taken to bucks Yes No

ii. Bucks brought to does Yes No

c. Artificial Insemination – goats on premises Yes No

d. Artificial Insemination – non-owned goats Yes No

e. I don't breed my goats Yes No

29. How many kids were born in the most recent season? _____

30. How many new goats from outside sources are introduced to the farm per year on average for new stock, breeding, etc.? _____

31. Are new goats obtained from:

a. Births from goats on the farm

b. Purchases at shows/fairs

c. Purchases from other breeders within the county

d. Purchases from other breeders within Indiana

e. Purchases from out-of-state breeders

32. Do you ever board your goats at another facility? Yes No

33. Do you board sheep, cattle or goats for others? Yes No

a. Do you take specific hygienic precautions when milking? Yes No

b. What precautions do you take (mark all that apply)?

i. Clean the teats prior to milking

ii. Pre-dip teats

iii. Post-dip teats

iv. Location specific for milking

v. Other

_____

_____

_____

_____

c. Where are your goats housed?

a. Mostly indoors

b. Mostly outdoors

c. Even split between indoors and outdoors

d. What are the goats flooring indoors made of?

a. Dirt

b. Concrete

c. Other _____

e. How often do you clean the goats bedding?

a. Less than 1x/month

b. Monthly

c. Every two weeks

d. Weekly

e. Daily

f. How do you dispose of the litter?

_____

_____

_____

g. Do you use any of the following to clean the stalls?

a. Detergents such as soap Yes No

b. Disinfectants such as bleach Yes No

c. Just water Yes No

d. Do not use anything Yes No

h. Do you use any pressure washers? Yes No

i. Do your goats have contact with sheep, goats or cattle on adjacent farms?

Yes No

j. Are there farms adjacent to your property with sheep, goats or cattle?

Yes No Don't Know

a. How many farms? _____

b. If yes, what type of farms?

i. Sheep

ii. Goats

iii. Cattle

k. Do you know if there are non-adjacent, nearby (within 1 mile) farms with sheep, goats or cattle?

Yes No Don't Know

a. How many farms? _____

b. If yes, what type of farms?

i. Sheep

ii. Goats

iii. Cattle

l. How often do your animals see a veterinarian?

a. Regularly – yearly

b. Regularly – 2x/ year

c. Regularly – 3x/year

d. Regularly - Quarterly

e. Regularly - Weekly

f. As needed

g. Never

m. What is your veterinarian's primary area of practice?
    a. Small animal medicine
    b. Food animal medicine
    c. Equine medicine
    d. Goat/Small ruminant medicine
    e. Mixed practice
    f. Uncertain

n. Do you routinely deparasitize your goats? Yes No
    a. If yes, what do you use (list all)?

    _____

    b. How often do you use it?
        i. Monthly
        ii. Every other month
        iii. Quarterly
        iv. 3x/year
        v. 2x/year
        vi. Annually
        vii. As needed
        viii. Never

o. Do you take any of the following precautions when working with goats:
    a. Wear gloves Yes No
    b. Wear coveralls that you remove before going home Yes No
    c. Wear a face mask Yes No
    d. Wear goggles Yes No

p. Do you take any of the following precautions when visiting another farm with goats:
    a. Wear gloves Yes No
    b. Wear coveralls that you remove before going home Yes No
    c. Wear a respirator/surgical mask Yes No

d. Wear eye protection Yes No

q. Do you routinely attend the births of your goats? Yes No

a. If yes, do you take any precautions when attending the births?

i. Wear gloves Yes No

ii. Wear dedicated coveralls Yes No

iii. Wear a surgical mask Yes No

iv. Wear a respirator Yes No

v. Wear eye protection Yes No

vi. Other

_____

r. Have you or someone in your family or employee ever gotten a flu-like illness during a kidding season? Yes No

a. If yes, was it diagnosed? Yes No

b. Diagnosis: _____

s. Do you ever consume raw milk from your goat(s)?

Yes No Rarely

If answer to 50 is Yes:

a. For how many years have you been consuming raw milk?

_____

b. On average, how much do you consume a day?

_____

c. On average, how much does your family consume a day?

_____

d. Do you also consume raw cow milk? Yes No

e. Do you acquire raw milk from any other location? Yes No

Where?

i. Store

ii. Neighbor

iii. Other

_____

f. What is the primary reason for why you consume raw milk?

i. Taste Yes No

ii. Allergies Yes No

iii. Convenience Yes No

iv. Health Yes No

v. Other:_____

_____

If answer to 50 is No:

g. What is the primary reason for why you do not consume raw milk?

_____

_____

_____

t. Do you ever consume pasteurized milk from your goat(s)? Yes No

a. What method do you use to pasteurize milk?

i. Vat pasteurization (30 minutes 145^{o}F/63^{o}C) Yes No

ii. High temperature short time (15 seconds 161^{o}F/72^{o}C)

Yes No

iii. Ultra Pasteurization (2.0 seconds 280^{o}F/138^{o}C)

Yes No

iv. Other:

Equipment: _____

Temperature: _____

Duration: _____

u. Do you think there are risks to people associated with owning goats?

Yes No

If yes, what are some of those risks?

_____

_____

_____

_____

53. Do you know of any diseases that can cause abortions in goats?

Yes No

If yes, can you tell me what some of those diseases are?

Yes No

_____

_____

_____

_____

v. Do goats carry any diseases that can be transmitted to people?

Yes No

If yes, Can you tell me what some of those diseases are?

Yes No

_____

_____

_____

_____

_____

55. Before speaking with us had you ever heard of a disease called Q-fever that is caused by a bacterium called *Coxiella burnetii*? Yes No

If yes, do you know if it can be transmitted to people?

Yes it can be transmitted to people

No it cannot be transmitted to people

I don't know

If it can be transmitted to people, how are people most likely to get the disease?

_____

_____

VITA

VITA

EDUCATION

Purdue University, West Lafayette, Indiana

Ph.D., Epidemiology and Public Health — December 2015

Thesis: Epidemiology of *Coxiella burnetii* in goats in Indiana

Advisor: Professor George E. Moore

Michigan State University, East Lansing, Michigan

Doctorate of Veterinary Medicine — May 1999

Bachelor of Science, Zoology — May 1995

Concentration in neurobiology and behavior

PROFESSIONAL EXPERIENCE

Research

Graduate Research Assistant — 2012- 2013

Epidemiology of Coxiella burnetii in Goats in Indiana

Additional Projects:

Herd prevalence of *Coxiella burnetii* in bulk tank samples from commercial herds in Indiana

Hellmann Lab, University of Notre Dame, Notre Dame, Indiana — 2004-2008

Assisted as a volunteer in various aspects of research projects related to butterflies as models of climate change

Teaching

GK-12, Purdue University, West Lafayette, Indiana and Tecumseh Junior High School, Lafayette, Indiana — Fall 2014

Disease Detection: Finding Undercover Agents When No One is Sick

Teaching Assistant**,** Purdue University, West Lafayette, Indiana — 2014, 2015

Comparative Anatomy I Laboratory

Comparative Anatomy II Laboratory

Tutor, Applications and Integrations, Purdue University, West Lafayette, Indiana
Spring 2013, 2015

Guest Lecturer, Purdue University, West Lafayette, Indiana
Introduction to Epidemiology Fall 2015
Public Health in Practice Spring 2012, 2013

Adjunct Professor, Brown Mackie College, South Bend, Indiana Fall 2011
Veterinary Pharmacology
Exotic and Laboratory Animal Medicine
Parasitology and Microbiology

Clinical Practice
Associate Veterinarian, Roseland Animal Hospital, South Bend, Indiana 1999–2011

Service
Reviewer
Veterinarni Medicina Fall 2015
Preventive Veterinary Medicine Fall 2015

Tutor, Boiler Vet Camp, Purdue University College of Veterinary Medicine Summer 2015

Graduate Student Delegate, Graduate Studies Committee, Department of Comparative Pathobiology, Purdue University College of Veterinary Medicine 2013-2015

Alternate Graduate Student Delegate, Grievance Committee, Department of Comparative Pathobiology, Purdue University College of Veterinary Medicine 2012-2015

PUBLICATIONS AND PRESENTATIONS
Publications
Bauer, Amy, Olivas, Sonora, Cooper, Maria, Hornstra, Heidie, Keim, Paul, Pearson, Talima, Johnson, April. Estimated herd prevalence and sequence types of Coxiella burnetii in bulk tank milk sampled from commercial dairies in Indiana. *BMC Veterinary Research* 2015, 11:186. doi: 10.1186/s12917-015-05

Bauer, A.E., Roberts, F.D. "Veterinarian-client discourse." *The International Encyclopedia of Language and Social Interaction*, First Edition. Karen Tracy (General Editor), Cornelia Ilie and Todd Sandel (Associate Editors). © 2015 John Wiley & Sons, Inc. Published 2015 by John Wiley & Sons, Inc.

Presentations

Weng, Hsin-Yi and Bauer, Amy (2015) *Knowledge of and protection practices against zoonotic diseases among goat owners in Indiana.* Oral presentation at the American Public Health Association Annual Meeting; November 3, 2015, Chicago, IL

Bauer, A.E., Olivas, S., Hornstra, H., Keim, P., Pearson, T., Johnson, A.J. (2015) *Sequence types of Coxiella burnetii in bulk tank milk samples from Indiana dairies.* Oral presentation at the Indiana Academy of Science Annual Meeting, March 21, 2015, Indianapolis, IN

Bauer, A.E., Cooper, M. and Johnson, A.J. (2013) *Prevalence and geographic distribution of Coxiella burnetii in bulk tank milk samples from Indiana dairies: a zoonotic pathogen of human and animal health importance.* Oral presentation at the Indiana Academy of Science Annual Meeting; March 2, 2013, Indianapolis, IN

Bauer, A.E., Cooper, M. and Johnson, A.J. (2012) *Herd prevalence and geographic distribution of Coxiella burnetii in cattle bulk tank milk samples in Indiana*. Oral presentation at the Conference of Research Workers in Animal Disease; December 3, 2012, Chicago, IL

Posters

Olivas, Sonora, Bauer, Amy, Hornstra, Heidie, Keim, Paul, Johnson, April, Pearson, Talima. *Genetic and geographic distribution differences of Coxiella burnetii among U.S. milk samples with a focus on Indiana.* The Annual Meeting of the Arizona-Nevada branch of the American Society for Microbiology; April 24, 2015, Flagstaff, AZ (Check date with Sonora)

Bauer, Amy E., Olivas, Sonora, Hornstra, Heidie M., Keim, Paul, Pearson, Talima R., Johnson, April J. *Sequence typing of Coxiella burnetii DNA from commercial bulk tank milk samples in Indiana*. Purdue University College of Veterinary Medicine Phi Zeta Research Day; April 14, 2014, West Lafayette, IN

Grants and Awards

Purdue University Graduate School Summer Research Grant Summer 2015

Purdue Service Learning Grant Program for Community Service/Service Learning Projects Fall 2014
Disease Detection: Finding Undercover Agents When No One is Sick

Indiana Academy of Sciences Fall 2012
Prevalence and Geographic Distribution of Coxiella burnetii in Bulk Tank Milk Samples from Indiana Dairies: A Zoonotic Pathogen of Human and Animal Health Importance